CELLULAR INSULIN ACTION IN METABOLIC DISEASE

Roy Taylor

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We should accustom ourselves to the idea that a primary deficiency of insulin is only one, and then not the commonest cause of the diabetic syndrome.

H. Himsworth
1949

ABSTRACT

The control of carbohydrate metabolism depends not only upon the concentrations of circulating hormones but also upon the responsiveness of target organ tissues. Following the demonstration of insulin receptors upon circulating monocytes, it became widely believed that the insulin receptor status of the important target organ tissues was mirrored by peripheral blood monocytes. Clinical states of insulin resistance were thus categorised as being related to either receptor or post-binding defects by the results of monocyte insulin receptor assays. The studies described in this thesis were designed to examine the clinical relevance of monocyte insulin receptor status and to define the nature of peripheral tissue insulin resistance in hepatic cirrhosis, chronic renal failure, hyperthyroidism and brittle diabetes.

The adipocyte and monocyte insulin receptor assays were optimised with respect to accuracy, precision, level of non-specific binding and maintenance of steady state binding. The two assays were then used to examine normal and insulin resistant subjects. No correlation between the insulin receptor status of the two tissues could be demonstrated in any group of subjects studied. All subsequent studies were performed using adipose tissue as an example of a peripheral, insulin sensitive tissue.

Subjects with hepatic cirrhosis were found to be markedly insulin resistant in vivo and adipocyte insulin receptor number was decreased in the cirrhotic subjects, especially in those with alcoholic cirrhosis. Adipocyte resistance to insulin stimulation of lipogenesis was present regardless of aetiology. In chronic renal failure, insulin resistance was evidenced by fasting hyperglycaemia and impaired glucose tolerance in the presence of normal serum immunoreactive insulin levels. Adipocyte insulin receptor number was found to be decreased, but receptor affinity was supra-normal. This unusual insulin receptor status was associated with normal adipocyte insulin sensitivity. Continuous ambulatory peritoneal dialysis was found to be associated with improved blood glucose control and normal adipocyte insulin receptor affinity and insulin sensitivity.

In hyperthyroidism, adipocyte sensitivity to stimulation of lipogenesis and glucose transport was found to be normal although adipocyte insulin receptor number was markedly decreased. These findings suggest that thyroid hormones may modulate insulin receptor number and also that adaptation of the post-binding steps of insulin action prevents the theoretical consequence of decreased insulin receptor number in this condition. The presumed *in vivo* insulin resistance in hyperthyroidism appears to relate to hepatic rather than peripheral tissue abnormalities.

A group of subjects with brittle insulin-dependent diabetes were observed to have decreased adipocyte insulin receptor affinity and decreased sensitivity to insulin stimulation of lipogenesis compared with matched groups of stable insulin-dependent diabetic and normal subjects. These abnormalities were found to remit after periods of stable metabolic control achieved by continuous intraperitoneal or intravenous insulin infusion. Thus, tissue insulin resistance is unlikely to be the primary cause of the pronounced metabolic disturbances of brittle diabetes, although once present may exacerbate the clinical syndrome.

In summary, the work presented in this thesis demonstrates that monocytes may not be used as indicators of the insulin receptor status of target organ cells, and that separate factors must control receptor and post-binding steps in cellular insulin action. The insulin resistance of chronic renal failure appears to be related to circulating inhibitors of insulin action, in contrast to that of hepatic cirrhosis and brittle diabetes in which binding and post-binding abnormalities of adipocyte insulin action were demonstrated.

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Finally, I thank Aileen, my wife, for enthusiastic support and for sharing the excitements and frustrations of research.

DECLARATION

The adipocyte and monocyte assays were carried out by myself with the exception of the glucose transport studies and the last six adipocyte studies with which Mr Clive Hetherington assisted. Dr Robert Heine supervised the first eight euglycaemic clamps. Hormone radioimmunoassay and blood sampling from cannulae during the latter part of approximately half of the glucose tolerance tests was performed by others. All of the methodological work upon the insulin binding and action studies was performed by myself, as were all adipose tissue biopsies. The design of the clinical studies, the recruitment of subjects, and all other aspects of the studies apart from those specifically mentioned above were carried out by myself. The composition of this thesis is entirely my own work.

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CHAPTER ONE

INTRODUCTION

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GENERAL BACKGROUND

The discovery of insulin by Banting and colleagues (1922) revolutionised the management of diabetes mellitus and reinforced the concept that the diabetic syndrome resulted from hormone deficiency. Over the subsequent decade the dramatic effects of insulin therapy were self-evident, although clinical observation suggested that insulin did not bring about a uniform response in all cases of diabetes mellitus (Orator 1927, Falta & Boller 1931). In healthy men, individual variation in the response to exogenous insulin was documented (Himsworth 1935). Shortly afterwards, diabetic subjects were found to differ markedly in glycaemic response following intravenous insulin administered at the same time as oral glucose (Himsworth & Kerr 1939). This test divided the diabetic subjects into two distinct groups, those sensitive to insulin and those relatively insensitive to insulin. Himsworth observed that the insulin insensitive subject was typically obese and often had arteriosclerotic complications. In such subjects, the disease was less likely to have had a sudden onset. It was also shown that insulin sensitivity could be modified by variation in the carbohydrate content of the diet and by treatment of the insulin insensitive state of Cushing's syndrome. This early suggestion that the response to insulin could be affected by changes in hormonal or metabolic state stimulated the debate about the aetiology of the glucose intolerance observed in chronic liver disease (Naunyn 1906, Rankin et al 1953), uraemia (Linder et al 1925) and states of steroid hormone, growth hormone and thyroid hormone excess (Conn & Fajans 1956, Cerasi & Luft 1964, Hales & Hyams 1964).

Following the development of radioimmunoassay for insulin, demonstration of raised serum immunoreactive insulin levels in chronic liver disease (Megyesi et al 1967), uraemia (Hampers et al 1968), Cushing's syndrome (Pupo et al 1966), acromegaly (Beck et al 1965) and thyrotoxicosis (Doar et al 1969) suggested that insulin insensitivity rather than insulin deficiency was the primary cause of the impaired glucose tolerance associated with these conditions. The abnormal response to insulin was variously attributed to circulating insulin antagonists (Ensinck et al 1965) or to altered substrate

supply (Randle et al 1966). Accumulation of detailed information upon cellular hormone receptors provided impetus for the concept that insulin receptor abnormalities could account for states of insulin insensitivity. This hypothesis became a central theme of research into insulin action (Freychet et al 1971a, Olefsky 1976, Beck-Nielsen et al 1978).

HISTORY OF THE CELLULAR RECEPTOR CONCEPT

The concept of specific receptors for chemical messengers was first postulated by Langley (1878 & 1905) who suggested that drugs and hormones did not act directly upon biological processes but that some receptive substance mediated the cellular effects. Ehrlich (1913) extended the concept, naming the specific chemical groupings "receptors". A direct relationship between the number of occupied receptors and intensity of drug action was hypothesized by Gaddum (1926) and Clark (1937). The observation that the maximal response to a drug could be obtained by occupation of only a proportion of drug receptors (Stephenson 1956) led to a more dynamic picture of ligand-receptor interaction. Monod, Wyman and Changeux (1965) and Rubin and Changeux (1966) proposed that the properties of a receptor could be modified by binding of ligand to neighbouring receptors.

Advances in hormone receptor physiology were largely of a theoretical nature until the advent of radio-labelled hormones of high specific activity. The first such compound was tritiated oestradiol which was shown to be retained specifically in the nucleus of target tissues (Glasscock & Hoekstra 1959, Edelman et al 1963). It was later shown that the steroid was binding in a stereospecific manner to a protein which could be isolated by density gradient centrifugation (Toft et al 1967). Intracellular receptor proteins for the major steroid hormones had been identified by 1970, and pathological states such as testicular feminisation in mice and male psuedohermaphroditism in humans were soon associated with steroid receptor abnormalities (Bullock & Bardin 1972).

It became apparent at an early stage that peptide hormones

differed from steroid hormones in that the cell plasma membrane was most probably the major site of action (Levine 1965, Pastan et al 1966). Cuatrecasas (1969) reported that insulin, bound covalently to Sepharose beads too large to be endocytosed, could increase glucose oxidation and inhibit lipolysis in isolated fat cells. Although this work was shown to be technically flawed (Davidson et al 1972, Fritz 1972), other experimental evidence was soon forthcoming (Jarrett & Smith 1974).

Radio-labelled insulin had been used in the radioimmunoassay for insulin since 1960 (Yalow & Berson 1960) but uncertainty about the biological activity and binding behaviour of the iodinated molecules limited the applicability of such preparations for binding studies. In 1971, a technique for the preparation of a mono-iodinated insulin was described (Freychet et al 1971b). This compound was almost as active as native insulin in stimulating glucose oxidation in isolated fat cells. With the development of a satisfactory probe of insulin receptor function, detailed exploration of the characteristics of the insulin receptor in different tissues could be undertaken.

INSULIN RECEPTOR PHYSIOLOGY

a)Molecular structure of the insulin receptor

Early work by Cuatrecasas (1973) established that the insulin receptor was a membrane associated glycoprotein of molecular weight around 300,000 Daltons. Jacobs and colleagues (1979) and Pilch and Czech (1979) demonstrated that the receptor was composed of subunits linked by disuphide bonds. Further work established that the insulin receptor consists of two α subunits (125,000 Daltons) which carry the insulin binding sites and two β subunits (95,000 Daltons) which are involved in transmission of the insulin signal (Roth & Cassel 1983).

b)Specific binding

The universal property of receptors is their ability to recognise and bind a specific ligand in the presence of a vast excess of other molecules. Insulin analogues inhibit the binding of ^{125}I-insulin to a degree corresponding to their structural similarity to

insulin itself. Thus desnonapeptide proinsulin, proinsulin and desoctapeptide insulin display 12%, 4% and 1% of the binding displacement activity of native insulin respectively, and these percentages correspond to the bioactivity of the analogues in rat adipocytes (Freychet et al 1974). Glucagon and other peptide hormones were found not to inhibit binding of ^{125}I-insulin (Freychet et al 1971, Cuatrecasas 1971a).

As insulin receptors were envisaged as being responsible for the binding of both labelled and unlabelled hormone, the finding that some binding of labelled insulin always occured even at extremely high native hormone concentrations required explanation. The concept of non-specific binding developed (Cuatrecasas 1971a). The capacity of the receptors is finite but the potential capacity for non-specific adherence of hormone to cell membrane or other biological material is theoretically non-saturable. Hence, when all the receptors are occupied by native hormone, the binding of labelled insulin to cells or membranes must be accounted for by "non-specific" binding. The binding observed in the presence of 10^{-5} molar unlabelled hormone concentration is subtracted from the total binding observed at any total hormone concentration in order to derive the specific binding fraction.

c) Kinetics

The insulin - receptor interaction in vitro was shown to be rapid and reversible (Cuatrecasas 1971b & 1972), characteristics that were predicted from the relatively rapid onset and cessation of the cellular action of insulin (Gliemann 1968). Dissociation of the adipocyte insulin-receptor complex was found to be a first order process and furthermore when labelled insulin was eluted from binding sites on fat cell membranes, both the hormone and the receptor retained their specific properties. The rates of association and dissociation were both found to be decreased at low temperatures, but as the latter was decreased to a greater extent, binding to isolated fat cell membranes was found to be higher at 5°C than 37°C (Cuatrecasas 1971b). Degradation of the labelled insulin, a slow process at low temperatures, complicated the results of binding experiments performed at 37°C. In particular, steady state binding to

isolated rat liver cells could not be achieved at 37°C (Freychet et al 1974) or to solubilized insulin receptors from rat liver membranes (Cuatrecasas 1972). Gammeltoft and Gliemann (1973) were, however, able to demonstrate steady state binding to isolated rat adipocytes at 37°C, possibly related to a lower total insulin degradation in this system (Gliemann & Sonne 1978).

d) Spare receptor concept

The classical pharmacological concept of receptor mediated biological effects was that of a direct relationship between receptor occupancy and biological effect. Thus a maximal response would be expected when all the receptors were occupied. However, Stevenson (1956) observed that there appeared to be more drug receptors than required for maximal response. Kono and Barham (1971) showed that this was also the case for insulin receptors. They observed maximal stimulation of glucose oxidation when only one-fortieth of the binding sites were filled and that a 98% decrease in binding capacity brought about by tryptic digestion did not reduce the maximum effect of insulin but increased the insulin concentration necessary for it to be achieved. The teleological significance of the presence of spare receptors was seen as allowing a greater sensitivity of target cells to low concentrations of hormone. Different tissues have been observed to require different degrees of receptor occupancy for a maximum response to insulin, from 2.5% in rat adipocytes (Kono & Barham 1971) to 35% in rat hepatocytes (Frank et al 1981).

e) Negative cooperativity

Early studies of insulin - receptor interaction yielded results suggestive of a homogenous species of insulin receptors, and it was assumed that the affinity was fixed (Cuatrecasas 1972). However, DeMeyts and colleagues (1973) demonstrated that as the occupancy of the insulin receptor increased, the affinity of the receptors decreased, resulting in acceleration of the dissociation rate of insulin from receptor. The precise physiological role of this phenomenon, referred to as negative cooperativity, remains unclear, but it could provide a mechanism for buffering the target cell against sustained elevations in circulating hormone levels and as an amplification of the response to low hormone concentrations. Negative

cooperativity provides a potential mechanism for regulation of receptor affinity, but changes in receptor number may also be brought about by incubation of cells in the presence of insulin (Gavin et al 1974). The in vitro phenomenon of downregulation has been extrapolated to explain observations of decreased cellular receptor number in both animals (Soll et al 1975, Freychet et al 1972) and man (Bar et al 1976).

Analysis of insulin binding data by the method of Scatchard (1949) yields curvilinear plots consistent with the concept of negative cooperativity. The shape of the Scatchard plot would also be consistent with the presence of two or more distinct classes of binding sites (Kahn et al 1974) and although this possibility remains, the balance of experimental evidence remains in favour of negative cooperativity between a homogenous population of receptors (DeMeyts 1980).

f) Internalisation

Following the demonstration of the linkage between specific binding of insulin and its degradation (Terris & Steiner 1975) it became clear that the insulin - receptor interaction was more complicated than a simple cell-surface bimolecular reversible reaction. Insulin conjugated to fluorescent lactalbumin was observed to bind to mouse fibroblasts diffusely at 4°C, but in clusters on the cell membrane at higher temperatures (Schlessenger et al 1978), raising possible parallels with the macroglobulin receptors which when occupied form invaginated clusters on the cell surface (Willingham et al 1979). Clustering of occupied receptor is theoretically attractive, providing a means of regulating the insulin receptor concentration and a mechanism for efficient adsorptive pinocytosis.

Autoradiographic techniques using ^{125}I-insulin unequivocally demonstrate that the hormone is internalised by hepatocytes. At steady state binding approximately one half of the insulin is bound to the hepatocyte cell membrane and the remainder has been internalised (Gorden et al 1978). In rat adipocytes, approximately 50% has been internalised at steady state binding (Olefsky et al 1982b). After internalisation, the hormone-receptor complex appears to fuse with lysosomes (Carpentier et al 1979). The receptor mediated

degradation of ligand is inhibited by agents such as ammonium chloride and choroquine which inhibit lysosomal enzyme action (Gordon et al 1980, Whittaker et al 1981a). After cleavage of ligand from receptor, the receptor recycles and returns to the cell membrane (Steer & Ashwell 1980). De novo receptor synthesis is not required for the continued appearance of receptor at the hepatocyte cell surface (Whittaker 1984). The processes of receptor clustering, internalisation and recycling are obviously vital to regulation of cellular receptor behaviour, but it would appear that they are divorced from the events leading to the intracellular manifestations of hormone action. When internalisation is impaired or when recycling is inhibited, insulin action is not prevented (LeCam et al 1979, Hammons & Jarrett 1979).

CLINICAL STUDIES OF INSULIN RECEPTORS

a) Techniques

The discovery by Gavin and co-workers (1972) of specific insulin binding to human leucocytes opened up the field of clinical investigation of insulin receptor physiology. They observed that the affinity of insulin binding to leucocytes appeared to be similar to that exhibited by hepatocytes and adipocytes. In addition, the degree to which insulin analogues could displace ^{125}I-insulin from the leucocyte receptor varied with the ability of the analogue to stimulate glucose oxidation in rat adipocytes (Freychet 1971a). Subsequent studies of leucocyte insulin binding in states of insulin resistance gave results suggestive of an abnormality of insulin binding, but the data were not unequivocal (Archer et al 1973). In 1975, Schwartz (1975) demonstrated that monocytes were responsible for the greater part of the insulin binding capacity of mixed leucocyte preparations. Expression of the observed binding data per unit number of monocytes, rather than total leucocytes, resulted in a clearer separation of binding results from normal and insulin resistant patients. Beck-Nielsen and colleagues (1977) confirmed that monocytes accounted for the major part of the insulin binding capacity of peripheral blood cells, and further demonstrated the relative insulin binding behaviour of lymphocytes, polymorphonuclear

leucocytes, erythrocytes and platelets. The discovery of specific insulin receptors on human erythrocytes attracted much interest as this offered a method for estimating insulin receptor status without venesection of large volumes of blood (Gambhir et al 1977). The erythrocyte insulin receptor appeared to display similar kinetic characteristics to that of rat hepatocytes and human monocytes.

Measurement of specific insulin binding to human adipocytes was achieved over a decade ago (Marinetti et al 1972, Olefsky et al 1974), although these pioneer studies were complicated by problems of high non-specific binding, uncertain cell viability and lack of steady state binding. The development of an improved method of isolating and handling human adipocytes allowed examination at physiological temperature and pH of steady state insulin binding (Pedersen 1982a).

b) Physiological regulation of insulin receptors

Diurnal changes have been observed in blood cell insulin binding (Beck-Nielsen & Pedersen 1978). They were abolished by starvation, and insulin receptors on monocytes and erythrocytes exhibited rapid down-regulation following food intake. Chronic changes in monocyte receptor number have been observed after hypercaloric and hypocaloric dieting (Beck-Nielsen et al 1978 & 1980). Isocaloric diets rich in fat or sucrose brought about a reduction in insulin binding to monocytes (Beck-Nielsen et al 1978). However, high-fibre, high-starch, low-fat diets increased monocyte and adipocyte receptor number (Ward et al 1982, Pedersen et al 1982b, Hjollund et al 1983a). During the menstrual cycle variation in erythrocyte insulin binding has been reported (Bertoli et al 1980), but the significance of this is unclear as adipocyte insulin binding remains constant (Pedersen et al 1982a) as does in vivo insulin sensitivity (Yki-Jarvinen 1984).

Acute physical exercise has been shown to increase insulin binding to blood cells from normal subjects (Pedersen et al 1980). Pregnancy has been reported to be associated with normal monocyte and erythrocyte binding (Pedersen et al 1981b, Tsibris et al 1980) but decreased adipocyte insulin binding (Hjollund et al 1983b). Conflicting data exist for insulin receptor status in elderly subjects, both normal (Fink et al 1983) and reduced (Bolinder et al

1983a) adipocyte insulin binding having been reported.

The results of clinical studies of insulin binding are summarised in Table 1.1.

CELLULAR INSULIN ACTION

Binding of insulin to its receptor on the cell plasma membrane is the first step in the cellular action of insulin. The subsequent events which lead to changes in intracellular enzyme systems are collectively described as the post-binding steps of insulin action. The process of insulin internalisation is too slow to account for the biological effects of the hormone (Denton et al 1981). In addition, antibodies to the insulin receptor may bring about all the biological effects of insulin (Kahn et al 1977, LeMarchand-Brustel 1978), suggesting that the insulin receptor itself carries the information to induce intracellular changes. The activation of the insulin receptor itself either by insulin or antibody would appear to be the event which initiates the sequence of events leading to the observed intracellular effects.

Plasma membrane transport of glucose was the first site of insulin action to be recognised (Crofford & Renold 1965). Insulin causes an increase in the maximum activity of the glucose carrier without affecting its affinity, but this effect cannot be reproduced in broken cell preparations (Czech 1980). Insulin appears to bring about the recruitment of carrier units from an intracellular pool, associated with the Golgi apparatus, to the cell membrane (Cushman & Wardzala 1980). In addition to this change, insulin has been shown to affect the activities of the enzymes responsible for its major actions on carbohydrate and fat metabolism (Denton et al 1981). In most cases, the effect of insulin upon enzyme is a dephosphorylation reaction. Changes in cyclic adenosine and guanine monophosphate have been studied intensively as possible mediators of insulin action (Hepp et al 1977), but it would appear that the small decrease of the former and the small increase of the latter are insufficient to provide a satisfactory mechanism (Kono & Barham 1973, Fain & Butcher

1976). Also, no insulin induced changes in cyclic nucleotide concentration in muscle cells have been observed (Tarui et al 1976). Early hopes for ionised calcium (Clausen & Martin 1977), hydrogen peroxide (Mukerjee & Lynn 1977) and a peptide second messenger (Larner et al 1979) have remained unsubstantiated.

The logistics of transmitting a signal from a relatively small number of plasma membrane insulin receptors to all the cytoplasmic and mitochondrial enzyme systems of a cell are formidable. To achieve the required amplification it has been postulated that a membrane associated protein kinase could phosphorylate many intracellular protein molecules (Denton et al 1981). This would be similar to the likely mode of action of epidermal growth factor receptors, binding to which activates a membrane bound protein kinase which phosphorylates itself and a range of other proteins (Cohen et al 1980). Recent work on insulin receptor phosphorylation has shown that the insulin receptor is indeed a protein kinase (Roth & Cassell 1983) and that insulin can induce receptor autophosphorylation in cell-free systems (Gazzano et al 1983). The immediate substrates for the receptor protein kinase remain uncertain, as it is able to bring about phosphorylation of tyrosine and serine residues on all possible substrates yet tested (DeMeyts 1984).

INSULIN RESISTANCE IN CLINICAL STATES

Insulin resistance may be considered to be present when normal concentrations of insulin produce a subnormal biological response. Thus the presence of insulin resistance in vivo is implied either by the coexistance of a raised plasma insulin concentration and euglycaemia or hyperglycaemia, or of normal plasma insulin concentration and hyperglycaemia. Quantification of the degree of insulin resistance has been attempted by subcutaneous insulin and oral glucose administration (Himsworth 1935), intravenous insulin tolerance tests (Beck-Nielsen 1978), combined glucose and insulin infusions (Harano et al 1978) and euglycaemic hyperinsulinaemic clamps (DeFronzo et al 1979). It is apparent that any one of four distinct biochemical steps could be responsible for cellular insulin

resistance: binding of insulin to its receptor, initiation of a transmembrane signal, generation of an intra-cellular signal and subsequent modification of the transport systems and enzymes that result in the ultimate biological effects of insulin. At the present time clinical studies can merely assess two components of insulin action: the binding reaction in terms of receptor number and affinity and secondly overall insulin action in terms of maximal insulin-induced response and sensitivity of the response to insulin. Attempts have thus been made to categorise insulin resistant states into those characterised by binding defects alone and those characterised by intracellular or post-binding defects.

The common clinical states associated with insulin resistance are obesity (Harrison et al 1976, Olefsky 1976), non-insulin dependent diabetes (Olefsky & Reaven 1977, Beck-Nielsen 1978) and insulin-dependent diabetes (Harano et al 1981, DeFronzo et al 1982). In obesity, decreased monocyte insulin receptor number has been demonstrated repeatedly (Archer et al 1973, Bar et al 1976, Beck-Nielsen 1978), only two studies having found normal monocyte insulin binding (Misbin et al 1979 & 1983). Adipocyte insulin receptor number has been shown to be decreased in obesity by most workers (Kolterman et al 1980, Pedersen et al 1981c, Olefsky 1976) but not all (Amatruda et al 1975). The early suggestion that reduced cellular receptor number explained the insulin resistance of obesity (Olefsky 1976) has been displaced by the finding of post-binding defects of insulin action both in adipocytes (Pedersen et al 1981c, Ciaraldi et al 1981) and in vivo (Olefsky et al 1982a). It has been postulated that a spectrum of cellular defects may be responsible for insulin resistance, from those only involving binding to combined binding and post-binding defects (Olefsky 1982a).

Receptor defects were inferred as the cause of the insulin resistance in non-insulin dependent diabetics following blood cell receptor studies (Olefsky & Reaven 1977, Beck-Nielsen 1978). Adipocyte insulin binding in non-insulin dependent diabetes has been observed to be low (Kolterman et al 1981, Rizza et al 1981) or normal (Bolinder et al 1982, Lonnroth et al 1983a, Kashiwagi et al 1983), but studies of insulin action in adipocytes from non-insulin

dependent diabetics have consistantly shown insulin resistance. Both basal and maximally insulin-stimulated rates of adipocyte glucose transport have been observed to be subnormal in non-insulin dependent diabetes (Ciaraldi et al 1982), suggesting the presence of post-binding defects of insulin action. In insulin-dependent diabetes, cellular insulin binding defects have been described although intracellular defects of insulin action appear to be of greater importance (Pedersen et al 1982c, Lonnroth et al 1983b).

Pathogenetic mechanisms for the insulin resistance remain obscure. In obesity, it has been proposed that consistant overeating provokes hyperinsulinaemia which causes binding and post-binding defects in cellular insulin action (Kahn 1980). Induction of post-binding defects in adipocytes after six hour insulin infusions in normal subjects has recently been described (Mandarino et al 1984). The primary defect in the induction of insulin resistance in non-insulin dependent diabetes has been postulated to be hypersecretion of insulin, and in insulin-dependent diabetes, peripheral hyperinsulinaemia brought about by subcutaneous insulin administration. Other clinical states including hepatic cirrhosis, thyrotoxicosis and chronic renal failure have been reported to be associated with insulin resistance. The cellular basis of the insulin resistance in these states is uncertain, and factors common to both these disorders and overt diabetes mellitus have not previously been explored.

AIMS OF THE PRESENT STUDIES

The present studies were undertaken to examine the pathophysiology of insulin resistance in disease states characterised by insensitivity to endogenous or exogenous insulin. In order to assess the utility of monocyte insulin binding as a tool for the indirect assessment of insulin receptor status in metabolically relevant tissue, a comparative study of monocyte and adipocyte insulin binding in normal and disease states was performed. Using the adipocyte as a model in which insulin receptor status and cellular insulin sensitivity could be determined simultaneously, the conditions of hepatic cirrhosis, chronic renal failure, hyperthyroidism and "brittle" diabetes were investigated.

	CELL TYPE STUDIED			REFERENCES
	ADIPOCYTE	MONOCYTE	ERYTHROCYTE	
Physiological				
High sucrose diet		↓		20
Low calorie diet		↑		21,23,324
Exercise		↑	↑	271
Pregnancy	↓	N	N	150,273,340
Old age	↓			33
	N	N		101,307
Associated with diabetes				
IDD (average control)	↓	N	N	213,269,270 277
IDD (poor control)		↑	↑	278
IDD (high fibre diet)		↑	N	276
NIDD (non-obese)	↓	↓		191,255,302
	N			32,212
Glibenclamide therapy		↑	N	22,151
Metformin therapy			↑	156,215
Infant of diabetic mother		↑		247
		N	N	273
Associated with other clinical states				
Obesity	↓	↓	↓	5,81,179, 189,190,211 251,274,356
	N	N		2,234
Cirrhosis	N	N	↓	31,108,136
	↓	↓		129,332
Chronic renal failure		N	↓	112,231,321
Cushings syndrome			N	249
Acromegaly		↓		240
Insulinoma		↓		148
Thyrotoxicosis	↓			9,334

TABLE 1.1 Results of insulin binding studies in various clinical states in man. Arrows indicate increased or decreased maximum specific insulin binding, and N denotes normal insulin binding.

CHAPTER TWO

CLINICAL AND LABORATORY METHODS

CONTENTS OF CHAPTER TWO

ADIPOCYTE METHODS

a) Adipose tissue biopsy

Subcutaneous adipose tissue was obtained from the upper lateral gluteal region between 0800 and 0900 hours after an overnight fast. Following antiseptic skin preparation, lignocaine (1% without adrenaline) was infiltrated intradermally to produce an area of anaesthesia approximately 6 cm by 3 cm. A horizontal incision 4 to 6 cm long was made, the skin was mobilised and a self-retaining retractor (Weisslander) was applied in order to expose the subcutaneous adipose tissue. A flat disc of adipose tissue weighing 2-6 grams was then dissected out. The subcutaneous tissue was approximated using interrupted chromic catgut sutures (2-0) and the incision was closed using a continuous sub-cuticular Vicryl suture (4-0). A pressure dressing was left in place for 48 hours. No wound infection nor other complication was observed following any of the biopsies. The procedure itself was found to be totally painless, and the sensations reported over the 24-48 hours following adipose tissue biopsy varied from no discomfort at all to a moderate ache lasting up to 36 hours. In no case was sleep disturbed nor analgesia required as a result of the biopsy.

Subcutaneous adipose tissue obtained at the time of elective abdominal surgery was used to develop and validate the adipocyte assays. Samples were taken only from metabolically normal patients who were taking no drugs known to interfere with carbohydrate metabolism. All samples were taken at the start of the surgical procedure.

b) Adipocyte isolation

The adipose tissue sample was placed immediately in glucose-saline (glucose 5 mmol/l and sodium chloride 154 mmol/l) and was maintained at 37^{o}C. Within 30 minutes the tissue was finely chopped using scissors and the tissue fragments were thoroughly washed in the glucose-saline solution. Each gram of tissue was incubated in 2 ml of collagenase buffer (see Appendix I) for 90 minutes at 37^{o}C in a shaking water bath (120 cycles per minute) as described by Pedersen

and colleagues (1981a). The resulting material was passed through a plastic mesh sieve in order to remove excessive fibrous tissue and subsequently through two nylon mesh plus cotton gauze filters mounted in syringe barrels. The cell suspension was then washed three times in glucose free 5% human serum albumin buffer (Appendix I). The washing procedure consisted of centrifugation for 20 seconds at 10 g, aspiration of the infranate using a 10 centimeter 17 gauge needle, addition of fresh glucose free buffer and resuspension of cells. After the final wash, the cells were resuspended at an approximate lipocrit (volume of cells divided by total volume of suspension x 100) of 40%.

The use of very sharp scissors for chopping the tissue sample was found to be essential to minimise cell lysis, especially with adipose tissue samples containing a high proportion of connective tissue. Initial problems with marked cell lysis were associated with the presence of detergent traces in the nylon mesh, and thorough pre-washing of the material in distilled water reduced the extent of lysis. Preliminary studies established that the intact adipose tissue sample could remain in the glucose-saline solution at 37°C for 90 minutes without development of cell lysis and without loss of insulin binding activity (0.83 and 0.86 % specific binding per 10 cm^2 cell membrane after 30 and 90 min respectively). Routinely, the intact tissue sample remained in the glucose-saline transport medium between 30 and 45 min.

c) Adipocyte quantification

The methods of Pedersen and colleagues (1981a) were adopted for adipocyte quantification. The cell suspension was repeatedly inverted to achieve even distribution of cells and a microhaematocrit tube was rapidly filled by immersion in the suspension. Four such tubes were separately filled, sealed and centrifuged at 10,000 g for two minutes. To allow rapid and even filling of the microhaematocrit tubes it was found necessary to transfer the cell suspension to a short, wide tube (6 ml volume). Considerable practice was required to achieve uniform suspension of the cells and thus reproducible estimations of lipocrit. The volume fraction of cell material was

measured for each microhaematocrit tube and the mean of the four determinations was used to calculate the dilution factors necessary to prepare aliquots of 5% and 40% lipocrit suspension as required. During the clinical studies, the coefficient of variation of the lipocrit procedure was found to be 2.5%.

A sample of 4µl of freshly prepared suspension was placed on a haemocytometer and covered with a plastic cover-slide on which a grid was marked. The diameters of 160 cells were measured under 200 fold magnification using a micrometer eyepiece which had previously been calibrated using a stage graticule. The coefficient of variation of cell sizing was found to be 2.0%. The cell volume ($\pi d^3/6$) and surface area (πd^2) were calculated for each individual cell using a computer programme and the mean of the individual cell volumes and surface areas were derived. As a standard expression of quantity of adipocytes, binding and insulin action data was expressed per square area of cell membrane, this being calculated from the mean cell volume, mean cell surface area, and the lipocrit. Adipocytes consist of a large deposit of lipid with a thin rim of membrane bound cytoplasm, and hence expression of metabolic events in terms of cell surface area may be more physiological than in terms of cell number, especially as mean cell size varies greatly both within one individual and between individuals (Pedersen et al 1981a).

d) Adipocyte insulin binding

A homogenous preparation of insulin monoiodinated at the A_{14} position was used for the binding studies in order to avoid variability in binding secondary to the different properties of the A_{14} and A_{19} labelled isomers (Gliemann et al 1979). Insulin binding to adipocytes* was measured by incubating 300 µl adiquots of cell suspension (5% lipocrit) with 100 µl A_{14} labelled monoiodoinsulin (final concentration 4 - 16 pmol/l) and 100 µl buffer or unlabelled insulin (final concentration 120 - 21,000 pmol/l) in duplicate at 37°C in a HEPES buffer (see Appendix I). The binding was stopped by adding 10 ml chilled NaCl (154 mmol/l). Silicone oil (1 ml; density 0.97 gram/ml) was layered on the surface and the tubes were spun at 1,000 g for three minutes. The cell pellet was harvested using a pipe

* (defined as that amount of insulin associated with cells and expressed in terms of the total amount of tracer insulin present)

cleaner as described by Gliemann and Sonne (1978). Initially, very low specific binding was observed, and examination of the chemical and physical conditions of the incubations were undertaken.

Crude collagenase preparations from two different sources were compared by disaggregating portions of the same adipose tissue sample, and no major differences were found in specific insulin binding at 15, 45 and 90 min (0.65 vs 0.56, 0.83 vs 0.90, and 0.69 vs 0.74 % per 10 cm^2 cell membrane for Worthington and P-L Biochemicals products respectively). Non-specific binding was similar after use of each collagenase preparation (1.2 vs 1.3 % of maximum tracer binding respectively). All subsequent studies were performed using a single batch of P-L Biochemicals crude collagenase.

The effect of different types of albumin upon measured insulin binding was examined. Collagenase digestion and washing of the cells was carried out in human serum albumin, and binding was assessed in buffers containing either highly purified human serum albumin or Fraction V bovine serum albumin extensively dialysed and charcoal treated in the laboratory. Specific insulin binding was higher in the presence of human serum albumin (0.58 vs 0.41 % per 10 cm^2 cell membrane) and it was noted that the larger adipocytes appeared to lyse in the presence of bovine serum albumin (mean cell diameters after incubations 92 vs 82 μ; no difference in the distribution of smaller cells in the two preparations). Similar results were obtained when all incubations and washes were performed in human serum albumin or highly purified bovine serum albumin (Hoechst) (1.64 vs 0.88 % specific binding per 10 cm^2 cell membrane; mean cell diameters 139 vs 91 μ respectively). Human serum albumin was used in all buffers in subsequent assays.

The possibility of cell damage induced by vigorous shaking during the binding incubation was examined by carrying out parallel binding studies on cells shaken at 130 cycles per min at maximum stroke length and on cells shaken twice by hand during the incubation. No excess cell lysis was noted after vigorous shaking and maximum specific insulin binding was not reduced (1.08 vs 0.99 % per

10 cm^2 cell membrane with and without vigorous shaking respectively).

Dinonyl phthalate was used to separate the cells from medium in preliminary binding experiments, but it was found that the cell mass was more cohesive and easier to harvest when silicone oil was used. To ensure complete collection of cells through the oil the speed of centrifugation was increased from 800 to 1,000 g. This caused breakage of over 50% of the polystyrene tubes used hitherto, and the problem was circumvented by the use of polypropylene tubes.

During the phase of development of the adipocyte methods, the observed percentage insulin binding increased steadily, and after incorporation of all the modifications, steady state binding could be demonstrated (Figure 2.1). Binding assays were routinely stopped at 60 minutes. Specific binding* was calculated by subtracting the binding observed in the presence of 10 μmol/l unlabelled insulin from the total binding for each insulin concentration. The non-specific binding averaged 1.5 ± 0.2% of cell bound insulin at a tracer concentration of 16 pmol/l. The intra-assay coefficient of variation of specific binding at a tracer concentration of 4 pmol/l was 2.3%. Scatchard analysis was performed using a computer program which depended upon non-linear regression for derivation of parameters (Scatchard 1949).

e) Insulin Degradation

Degradation of A_{14}-labelled monoidoinsulin under the conditions described for insulin binding was assessed after 60 and 120 minutes by addition of 200 μl of trichloracetic acid (12%) to a 200 μl aliquot of the incubation medium. The precipitate was separated by centrifugation at 2,000 g for three minutes and the ratio of ^{125}I in the supernate to ^{125}I in the precipitate was determined. Degradation was assessed in the presence of tracer insulin (16 pmol/l) alone and in the presence of tracer insulin together with an excess of cold insulin (10 μmol/l). Degradation assessed by this method reflects both intracellular and extracellular (medium) degradation. As the latter is not inhibited by high concentrations of insulin, consideration of the percentage degradation both in the presence and absence of unlabelled insulin allows assessment of cell-mediated, specific insulin degradation. The amount of insulin degraded during a binding assay averaged 1.4 ± 0.1% of total radio-labelled insulin and

* (that is, percentage of tracer insulin associated with receptors according to the theory expounded on page 15)

hence was not likely to affect measurements of steady state binding.

f) Insulin stimulation of lipogenesis

Triplicate 200 µl aliquots of adipocyte suspension (5% lipocrit) in a glucose-free HEPES buffer (Appendix 1) were pre-incubated at 37°C for 45 min with or without increasing amounts of insulin. D-(U-^{14}C)-glucose (0.4 µCi; final concentration 6 µmol/l) was added and the incubation was continued without shaking for 90 minutes. The reaction was stopped by adding sulphuric acid (200 µl; 1 mol/l) and the tubes were allowed to stand for 60 minutes before the total lipid fraction was separated using the procedure of Dole and Meinertz (1960). The time course of lipogenesis is shown in Figure 2.2. The amount of D-(U^{14}-C)-glucose incorporated into lipid for each triplicate set of tubes was calculated and expressed as pmol glucose incorporated per 90 min per 10 cm^2 adipocyte membrane. The intra-assay coefficients of variation for basal and maximally insulin stimulated lipogenesis were 7.4 and 4.6 % respectively.

g) Adipocyte glucose transport

D-(U-^{14}C)-glucose was used to assess adipocyte glucose transport (Pedersen & Gliemann 1981, Zeuzem & Taylor 1984). Aliquots (40 µl) of 40% adipocyte suspensions in glucose free Hepes buffer were pre-incubated at 37°C for 45 minutes with or without increasing amounts of insulin (0 - 18,000 pmol/l final concentration). D-(U-^{14}C)-glucose (12 µl; final concentration 20 µmol/l) was added and the contents of the tube were thoroughly mixed. The incubation period was continued at 37°C for 20 seconds and was terminated by the addition of 2.5 ml chilled phloretin solution (0.3 mmol/l in sodium chloride 154 mmol/l). The cells were separated from extracellular medium within two minutes by spinning through silicone oil at 2,000 g. The cell pellet was harvested using a pipe cleaner and was added directly to scintillation fluid (5 ml). Extracellular trapped radioactivity was assessed by adding phloretin to triplicate aliquots of cells before injection of D-(U-^{14}C)-glucose. The radioactivity observed in these separated pellets was subtracted from all other observations. Basal glucose transport was assessed in quadruplicate and insulin

stimulated glucose transport in triplicate for each insulin concentration. The time course of basal and maximally-insulin stimulated glucose uptake is shown in Figure 2.2. The intra-assay coefficients of variation were 9% basally and 7% with maximal insulin stimulation. Results were expressed as pmoles of glucose transported per 20 seconds per 10 cm^2 cell membrane.

MONOCYTE METHODS

a) Development of methods

A major disadvantage of the established monocyte insulin binding assay was the need for 160 ml of blood to allow the separation of sufficient cells to perform one assay. The technique of single bag plasmapheresis appeared to offer the facility of isolating large numbers of leucocytes without depletion of red cell mass. The technique involves venesection of 500 ml of blood directly into a Fenwal triple pack which is then centrifuged at 1,000 g. The plasma and subsequently the buffy coat fraction are expressed into separate integral bags. The red cells and plasma are returned to the subject. The mean yield of leucocytes was 22.2 $\pm$ 6.4 x 10^7 (n=3) compared with 12.4 $\pm$ 1.9 x 10^7 (n=6) using simple centrifugation of blood. The percentage of monocytes in the leucocyte suspension was not greatly changed by the procedure (20 $\pm$ 1.8 vs 23.8 $\pm$ 1.7 % respectively). However, the procedure was found to be disliked by all subjects. Attempts were therefore directed to increasing the yield of leucocytes from smaller volumes of blood obtained by simple venesection. The first step of the separation procedure, centrifugation and harvesting of buffy coat cells, was found to leave approximately 60 % of leucocytes trapped in the red cell mass. Dilution of anticoagulated blood with an equal volume of phosphate buffered saline and centrifugation in 8 universal containers brought about an increased yield of leucocytes (9.9 $\pm$ 1.1 x 10^7 (n=6) vs 12.4 $\pm$ 1.9 x 10^7 (n=6) from 80 and 150 ml of blood respectively) without change in the proportion of monocytes present (23.3 $\pm$ 2.3 vs 23.8 $\pm$ 1.7 % respectively).

Monocytes account for over 80 % of the specific insulin binding of the mixed monocuclear cell population isolated by Ficoll-Hypaque density gradient centrifugation (Beck-Nielsen et al 1977). To allow direct measurements upon monocytes, attempts were made to isolate pure monocyte preparations. Loos and colleagues (1976) reported that monocytes and lymphocytes could be separated by discontinuous density gradient centrifugation. Mononuclear cell suspensions were centrifuged over Ficoll-Hypaque in density layers of 1.055, 1.062 and 1.080 gram/ml. The calculated density of each solution was confirmed using an Abbey refractometer. The results of the separation of cells into light (density >1.055<1.062 grams/ml) and heavy (density >1.062 <1.080 grams/ml) cells were assessed by cell counting in a haemocytometer and non-specific esterase staining for estimation of the percentage of monocytes present (Horwitz et al 1977). The yield of monocytes was low by this procedure although monocyte enrichment was observed (Table 2.1). Substantial numbers of monocytes were found in the heavy cell band. Loos and colleagues assessed the separation of monocytes and lymphocytes using electronic cell sizing and indirect measurement of phagocytosis and hence may have demonstrated merely that large mononuclear cells and activated macrophages have a lower density than small mononuclear cells and quiescent monocytes. In view of the poor yield and relatively poor separation of monocytes, the technique was not examined further.

Monocytes may be differentiated from lymphocytes by their capacity to adhere to inert surfaces (Brodersen & Burns 1973). Aliquots of mononuclear cell suspensions in Medium 199 with 10 % foetal calf serum were incubated in plastic Petrie dishes on ice for 60 min. The adherent cells were displaced by pipette aspiration and expulsion. Less than 10 % of the mononuclear cells were found to adhere and be displaced by this method, and no non-specific esterase staining could be demonstrated in the adherent cell population on three occasions, although the cells appeared to be mainly of monocyte morphology on phase-contrast microscopy. Further assessment of this method of monocyte isolation was not undertaken because of the uncertainty surrounding the effect of the procedure upon the staining properties of the cells and possible effects of metabolic changes

associated with cell attachment upon monocyte insulin binding.

b) Cell isolation

Venous blood samples (80 ml) were obtained from fasting subjects between 0800 and 0900 hours, were anticoagulated with E.D.T.A. and were immediately placed on ice. The blood was diluted 1:1 with phosphate buffered saline (pH 7.4) to maximise the yield of buffy coat cells obtained by centrifugation at 500 g for five minutes. Mononuclear cells were isolated by density gradient centrifugation of the buffy coat cell suspension using Ficoll/Hypaque (density 1.077 gram/ml), and were subsequently washed three times in phosphate buffered saline. The cells were resuspended in 4 ml of the assay buffer (see Appendix I). Cell counting and assessment of viability were carried out in a haemocytometer using Trypan blue as a vital stain. The coefficient of variation of cell counting was found to be 3.5% and viability was always greater than 98%. The percentage of monocytes in the mononuclear cell suspension was determined using a non-specific esterase stain (Horwitz et al 1977). The accuracy of this estimation directly affects the accuracy of the assay itself, observed binding being corrected to be expressed per 10^6 monocytes. A series of preparations were independently assessed by a consultant haematologist and the Spearman rank correlation coefficient was found to be 0.78, only 3 of the 19 readings differing by more than 5%. The mixed mononuclear cell preparation contained 15-65 x 10^6 cells per ml, of which 12-33% were monocytes, 67-88% were lymphocytes and <1% were polymorphonuclear leucocytes.

c) Monocyte insulin binding

Insulin binding to monocytes was measured at 20°C by incubating 150 µl aliquots of mononuclear cell suspension with 50 µl of A_{14}-monoiodoinsulin (final concentration 10-40 pmol/l) and 50 µl of buffer or unlabelled insulin (final concentration 0-1,000 nmol/l) as described by DeMeyts (1976). The incubation was terminated by the addition of 1 ml chilled normal saline, centrifugation, washing of the cell pellet with chilled saline (154 mmo/l) and aspiration of the supernatant. The cell associated radioactivity was assessed by cutting off and counting the tip of each microfuge tube. The assay

was originally carried out in 10 ml tubes and the cell suspensions were transfered to 3 ml polystyrene tubes for centrifugation, washing and counting. The polystyrene tubes were found to bind insulin, and this artefact increased the observed non-specific binding to over 15 %. The non-specific binding was decreased to 4.5 $\pm$ 0.5 % (n=6) by performing the entire assay in 1.5 ml polypropylene tubes, and the error inherent in a transfer step was avoided.

The time course of insulin binding is shown in Figure 2.1. Specific binding was calculated by subtracting the percentage binding observed in the presence of 10 μmol/l insulin from the percentage total binding for each insulin concentration, and was expressed as percentage specific binding*/per 10^6 monocytes. Less than 2% of total A_{14}-monoiodoinsulin was degraded during the assay. The intra-assay coefficient of variation was 4.2% and the inter-assay coefficient of variation, as assessed by measuring insulin binding to monocytes from one individual on seven occasions over a six month period, was 8.4%.

IN VIVO STUDIES

a) Glucose tolerance tests

Glucose (75 grams as 375 ml degassed Lucozade) was administered orally over a 5 minute period between 0800 and 0900 hours after an overnight fast. Patients remained sitting throughout the test and no smoking was permitted. Blood samples were taken via a plastic cannula inserted into an antecubital fossa vein under local anaesthesia.

b) Euglycaemic hyperinsulinaemic clamps

After an overnight fast, three venous cannulae (Venflon) were inserted. One cannula, in an antecubital vein, was used for intermittent blood sampling for hormone and metabolite levels and was flushed with saline (154 mmol/l) after use. A second cannula, inserted in a retrograde fashion and more distally on the same arm was used for continuous sampling by a computer controlled glucose infusion system (Biostator). Arterialization of venous blood was achieved using the heated hand technique (Abumrad et al 1981). All

* (that is, percentage of total tracer insulin, which is bound to receptors)

infusions were administered via the contralateral arm. A period of one hour was allowed to elapse before starting the infusions in order to complete calibration of the Biostator and to achieve stability of the glucose sensor response. An infusion of insulin in polygeline (Haemaccel) at a rate of 50 mU/kg/h was administered and glucose was infused at a rate determined by the Biostator in order to maintain the blood glucose at 0.2 mmol/l below the fasting level (DeFronzo et al 1979). The glucose disposal rate from 60 - 120 min was determined from the total glucose infused during this period.

c) Glucose/insulin infusion

Glucose/insulin infusions were performed by the method of Harano (1978) but without somatostatin infusion as this has been shown not to influence the results (Heine et al 1982). Glucose (6 mg/kg/min) and insulin (50 mU/kg/h) were infused at constant rates for 150 minutes and venous samples were taken from the contralateral arm every 15 minutes for blood glucose, blood metabolites, serum I.R.I. (immunoreactive insulin) and serum C-peptide estimations. Steady state blood glucose was calculated as the mean of the observed concentration between 120 and 150 minutes and the mean coefficient of variation during this period was 4.0 $\pm$ 0.6 %.

d) Hormone and metabolite assays

Serum was separated and stored at -20°C. Immunoreactive insulin (I.R.I.) was assayed by the method of Soeldner & Sloane (1965). The intra- and inter-assay coefficients of variation were 3.6 and 7.3 % respectively and proinsulin displayed 50 % cross-reactivity in the insulin assay. C-peptide was assayed by radio-immunoassay (Heding 1975) and the intra- and inter-assay coefficients of variation were 3.1 and 5.5 % respectively. Proinsulin was assayed by a modification of the method of Rainbow et al (1979) using flexible microtitre plates as solid phase and ^{125}I labelled mouse monoclonal anti-rabbit immunoglobulin. Proinsulin from human pancreas was extracted and calibrated for use as previously described (Rainbow et al 1979). The specificity of the proinsulin assay was 100 % for circulating proinsulin ($65A_1$ and 32-33 split isomers) and the intra- and inter-assay coefficients of variation were 5.8 and 10.0 %

respectively. Blood glucose was measured using a Yellowsprings analyser within 30 minutes of sampling, delay in analysis having been shown to be important despite the presence of fluoride oxalate (Sidebottom et al 1982). Whole blood lactate, glycerol and 3-hydroxybutyrate were analysed by an enzymatic fluorimetric assay (Lloyd et al 1978) and the intra- and inter-assay coefficients of variation for each assay were 2.7 and 4.2 %, 0.7 and 2.2 %, and 8.5 and 10.4 % respectively.

ETHICAL COMMITTEE APPROVAL

The protocol for each study was approved by the Newcastle Health Authority Ethical Committee. Before participation in any study was requested, the purpose, nature and potential adverse effects of each test were fully explained.

PRESENTATION OF DATA

All results are given as mean ± standard error of the mean. Statistical analyses were performed using Student's t test (paired or unpaired as appropriate) and linear correlation when data was normally distributed. The Mann-Whitney U test and the Spearman rank correlation were used to analyze skewed data.

	Total cell number $x10^6$	% monocytes	Total monocyte number $x10^6$
Initial cell suspension	68.6±14.1	25.0±4.7	16.8±4.4
Light cell population	13.2±5.0	41.0±1.2	5.3±2.0
Heavy cell population	23.8±5.5	13.3±1.8	3.1±0.8

TABLE 2.1 Effect of discontinuous density gradient centrifugation upon monocyte enrichment of mixed mononuclear cell suspensions. The yield of monocytes in the light cell band (density >1.055 <1.062 gram/ml) was 29.8 ± 4.1 %.

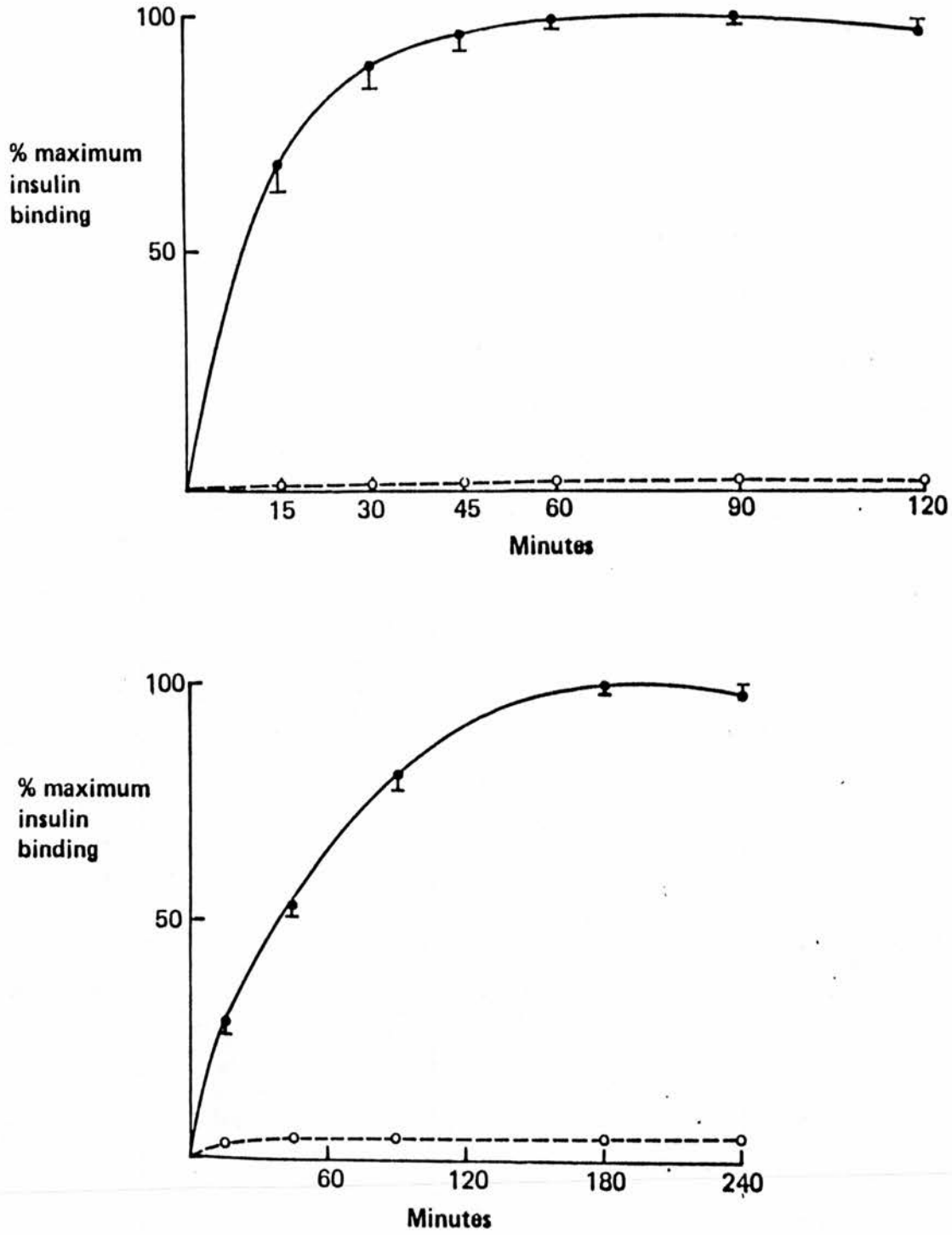

FIGURE 2.1 Time course of tracer insulin binding to human adipocytes at 37°C (top) and to peripheral blood monocytes at 20°C (bottom).

●——————● Specific insulin binding

o - - - - - o Non-specific insulin binding

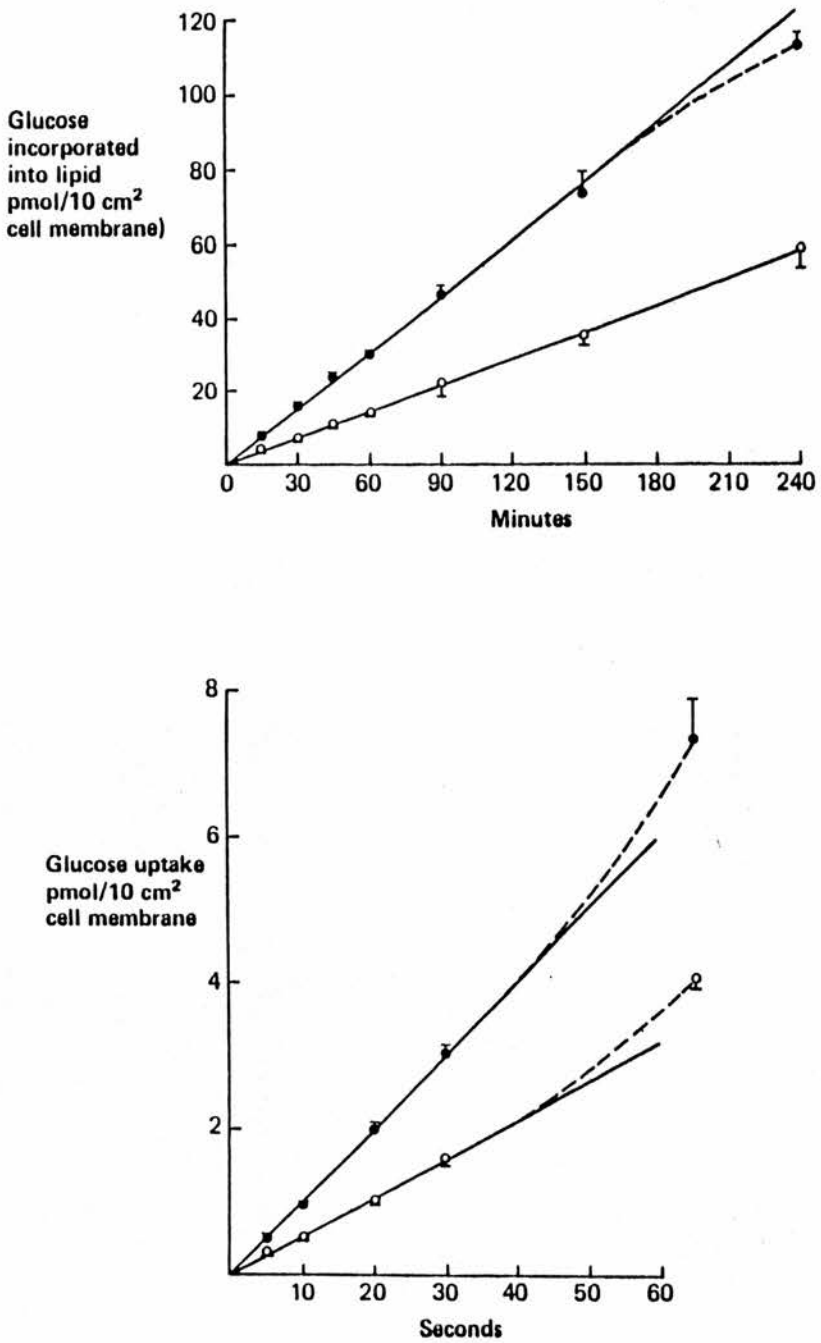

FIGURE 2.2 Time course of adipocyte lipogenesis (top) and adipocyte glucose transport (bottom).

o——o Non-insulin stimulated

●——● Maximally insulin stimulated (14 nmol/l)

CHAPTER THREE

THE RELATIONSHIP BETWEEN ADIPOCYTE AND MONOCYTE INSULIN BINDING

INTRODUCTION

The major objective of most clinical studies of insulin receptors has been to examine the role of insulin receptor abnormalities in the pathogenesis or pathophysiology of an insulin resistant condition. It is axiomatic that for studies to be relevant to clinical effects, the receptor status of the major insulin responsive tissues (liver, muscle and fat) should be examined. Changes in tissues which do not have a major metabolic response to insulin would be of interest only if they were to reflect changes in the insulin responsive tissues. Shortly after specific binding of insulin to mononuculear blood cells was first described (Gavin et al 1972) it was suggested that insulin binding to such cells might reflect insulin binding to metabolically important cells (Archer et al 1973). Since that time it has been widely believed that blood cell insulin binding mirrors the changes in target organ tissues. A small number of studies are quoted as supporting this hypothesis. Soll and colleagues (1973) demonstrated that thymic lymphocytes, adipocytes and liver membranes from obese mice bound less insulin than comparable cells or tissue from lean mice. Olefsky (1976) presented data which suggested a correlation between insulin binding to monocytes and adipocytes in a mixed group of normal and obese subjects. However, no evidence for a relationship between insulin binding to the two cell types in normal subjects was found, and the relative behaviour of monocytes and adipocytes in clinical states other than obesity remained uncertain.

In order to clarify the role of monocyte insulin receptor assays in clinical research, the relationship between insulin binding to monocytes and insulin binding to adipocytes was examined in normal subjects and in subjects with disorders of carbohydrate metabolism.

SUBJECTS AND PROTOCOL

Binding studies were carried out on thirteen healthy subjects (aged 28 to 71 years, 101 to 135% ideal body weight) recruited from the hospital staff, thirteen patients with insulin-dependent diabetes (16 to 28 years, 95 to 133% I.B.W.), eight thyrotoxic subjects (45 to 67 years, 80 to 125% I.B.W.) and fourteen subjects with histologically proven cirrhosis (29 to 71 years, 80 to 162% I.B.W.). Fasting fat biopsies and blood samples were obtained from any one individual within a three day period, during which time dietary intake remained constant.

RESULTS

No relationship was found between percentage maximum specific insulin binding to adipocytes (per 10 cm^2 cell membrane) and percentage maximum specific insulin binding to monocytes (per 10^6 monocytes) within the normal group ($R = -0.31$, $p = 0.31$), the cirrhotic group ($R = 0.30$, $p = 0.31$), the thyrotoxic group ($R = 0.04$, $p = 0.99$) or the diabetic group ($R = -0.23$, $p = 0.37$) (Figure 3.1). Pooling the data on all 48 individuals failed to demonstrate any correlation between insulin binding to the two cell types ($R = -0.17$; $p = 0.22$). The lack of correlation was not affected by expressing adipocyte binding data as maximum specific binding per 30,000 cells.

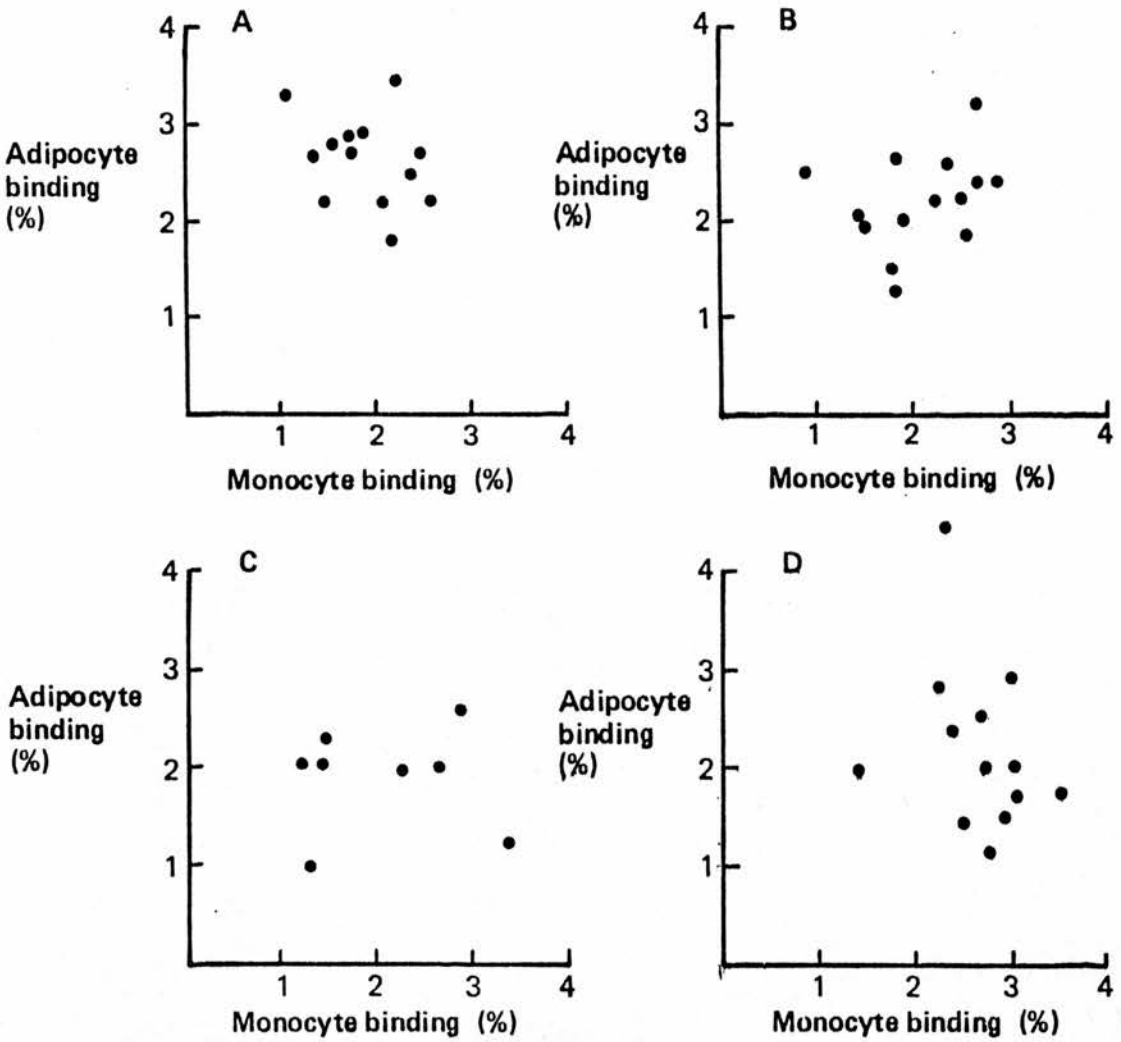

FIGURE 3.1 Relationship between percentage maximum specific insulin binding to adipocytes (per 10 cm^2 cell membrane) and to monocytes (per 10^6 cells):

A Normal subjects

B Cirrhotic subjects

C Hyperthyroid subjects

D Insulin-dependent diabetic subjects

DISCUSSION

The observed lack of correlation between insulin binding to monocytes and adipocytes might be regarded as surprising in view of the opinion prevailing in the insulin binding literature. However, examination of the papers quoted as supporting the concept of monocytes as reliable indicators of the insulin binding status of metabolically important cells shows the experimental evidence to be tenuous. Soll et al (1973) demonstrated that both thymic lymphocytes and adipocytes from obese mice bound less insulin than comparable cells from lean mice. This is often quoted first, as referring to circulating monocytes (Harrison et al 1976) and second, as showing a correlation between insulin binding to two cell types rather than a similar change seen in a specific pathophysiological state. Olefsky showed that insulin binding to both monocytes and adipocytes tended to be low in very obese subjects (>130% ideal body weight) but that there was no correlation between insulin binding to the two cell types in the normal subjects studied (Olefsky 1976). When the data from the obese and normal groups were pooled and analysed by linear regression a statistically significant result was obtained. The statistical validity of this analysis is questionable, and it is surprising to find the result (R=0.54) described as an "excellent correlation" (Bar et al 1976).

Although it would appear that insulin binding to fat and blood cells is decreased in obesity, our data would suggest that binding to the two different cell types does not necessarily change in the same direction in all pathological states. We observed low mean insulin binding to adipocytes but high mean insulin binding to monocytes in both the insulin-dependent diabetics and the cirrhotic patients. Pedersen and colleagues also failed to find a correlation between insulin binding to the two cell types using the same optimised adipocyte assay but a different monocyte assay in both normal subjects and insulin dependent diabetics (Pedersen et al 1982a, Pedersen & Hjollund 1982).

There are several possible reasons for the observed lack of correlation. It has been assumed in the past that monocytes are a

homogenous group of cells, although evidence for the existence of distinct sub-goups has been presented (Nathan et al 1976, Stuart 1977, Braciale et al 1982, Katz et al 1983). It is conceivable that changes may occur in the sub-group composition of the monocytoid group of cells in disease states which could of themselves affect the overall monocyte insulin binding capacity. A second factor which could account for lack of correlation between adipocyte and monocyte insulin binding is the differing environment to which the cell types are exposed. Adipose tissue is a fixed, peripheral tissue whereas circulating blood cells pass through the portal circulation and are thus exposed to pulses of insulin even in the fasting state (Blackard & Nelson 1970, Lang et al 1979). As insulin is the most potent known regulator of insulin receptors (Insel et al 1980), it is not surprising that in insulin dependent diabetes, a condition in which portal and systemic insulin levels are identical, a dislocation of any normal relationship between insulin binding to circulating cells and fixed peripheral tissue cells has been found in the present study and also by Pedersen & Hjollund (1982). In cirrhosis a similar mechanism may be operative as hepatic extraction of insulin is impaired (Johnston et al 1978).

The lack of correlation between insulin binding to monocytes and adipocytes in normal subjects which has been demonstrated in the present study and also by Pedersen and colleagues (1982a) is unlikely to be secondary to either of the two factors discussed above. Other influences must therefore act differentially upon individual cell types. Regulation of insulin receptor status may be mediated either directly upon the cytosolic and plasma membrane receptors, for example, insulin itself, or indirectly via the nucleus as in the case of thyroid hormones (Heise et al 1982). Interpretation of signals by the nuclei of different cell types could bring about varying effects upon insulin receptors.

The demonstration of specific insulin receptors on erythrocytes (Gambhir et al 1977) was also followed by the suggestion that insulin binding to these cells would reflect insulin binding to the cells of metabolically important tissues (Gambhir et al 1978). No convincing evidence has been provided to support this suggestion and the

subsequent finding that erythrocyte insulin receptor number depended heavily upon the age of individual cells (Kosmakos et al 1980, Eng et al 1980, Dons et al 1981a) did not deter the performance of many clinical studies (Gambhir et al 1980, Bhathena et al 1981, Fujita et al 1982, Lord et al 1983). Comparison between monocytes and erythrocytes show different characteristics of binding to the two cell types in a variety of conditions (Hjollund et al 1983c, Mandarino et al 1981, Dons et al 1981b, Spanheimer et al 1982). This remains the case even if the effect of erythrocyte age is removed (Camagna et al 1983). Similarly, the lack of correlation between insulin binding to erythrocytes and adipocytes (Pedersen et al 1981a, Benson et al 1982, Pedersen 1983) has been adequately revealed. This is not merely a reflection of differences in assay conditions for blood cells and adipocytes as performing adipocyte insulin binding under the conditions necessary for measuring erythrocyte insulin binding did not improve the correlation (Pedersen & Hjollund 1982).

The accuracy and precision of the binding assays are relevant to the interpretation of results, as a lack of correlation could merely reflect upon the assays. The coefficients of variation observed in the present study compare well with the few published assessments (Pedersen et al 1981a, Ward et al 1982). Non-specific binding usually accounts for 10 to 30% of total binding in both monocyte and adipocyte assays (Olefsky et al 1974, Beck-Nielsen et al 1980, DePirro et al 1981, Nankervis et al 1982), markedly higher than in the optimised assays used in this study. The level of specific binding to adipocytes observed in our normal subjects is not dissimilar to that recorded by Pedersen, but is higher than that observed by other workers (Olefsky 1976, Nankervis et al 1982) probably as a result of methodological improvements (Pedersen et al 1981a). Differences exist in the specific insulin binding to adipocytes from different anatomical sites (Bolinder 1983b) and hence it is important that adipocytes from only one site are used in comparative studies.

The relevance of monocyte binding to the insulin receptor status of the fixed peripheral tissues must be uncertain although there may

be some relationship to overall insulin sensitivity in certain clinical states (Olefsky & Reaven 1977, Beck-Nielsen et al 1978). In cirrhotic subjects a significant correlation between adipocyte (but not monocyte) insulin binding and both fasting serum insulin and in vivo insulin sensitivity has been observed in the present studies (Chapter 4). Insulin sensitivity measured in vivo is largely determined by muscle glucose disposal (DeFronzo et al 1981a) and therefore these findings suggest that the insulin sensitivity of muscle corresponds with adipocyte insulin binding at least in cirrhotic subjects. The two major insulin sensitive peripheral tissues, muscle and fat, are exposed to identical controlling influences, although it may not be assumed that adipocytes reflect the insulin binding status of myocytes or indeed hepatocytes. Examination of adipocyte insulin binding does provide information about an insulin sensitive tissue which may be examined in the light of in vitro studies of insulin action (Pedersen et al 1981c & 1982a, Pedersen & Hjollund 1982).

It remains possible that sequential studies of monocyte insulin binding in groups of individuals could reflect the direction of any change in receptor status in other tissues. This hypothesis has yet to be proven in man and preliminary results are not supportive (Mandarino et al 1984).

CHAPTER FOUR

HEPATIC CIRRHOSIS

INTRODUCTION

The association between chronic liver disease and impaired glucose tolerance has long been recognised (Naunyn 1906, Rankin et al 1953, Megyesi et al 1967). Naunyn (1906) hypothesized that pancreatic insulin secretion was not subnormal, some effect of the liver damage itself being responsible for the impairment of glucose tolerance. This was supported by observation of elevated serum levels of immunoreactive insulin in chronic liver disease of any aetiology (Megyesi et al 1967, Johnston et al 1982, Ghanem et al 1973) and by the finding of more marked hyperinsulinaemia in subjects with more severe impairment of glucose tolerance (Toccafondi et al 1977).

Coexistence of carbohydrate intolerance and hyperinsulinaemia in chronic liver disease suggests that insulin action is impaired. This has been confirmed by intravenous insulin tolerance tests (Collins et al 1970, West et al 1975), although precise quantitation of insulin resistance by this technique is complicated by the lack of steady state conditions and variability of the counter-regulatory hormone response. The pathogenesis of the insulin resistance could involve circulating antagonists of insulin action or defects in the tissue response to insulin. Circulating levels of the insulin counter-regulatory hormones have been intensively investigated. Basal growth hormone levels have been found to be elevated in most (Conn & Daughaday 1970, Muggeo et al 1979, Borzio et al 1981) but not all (Owens et al 1973, Kasperska-Czyzykowa & Rogala 1978) studies of subjects with established cirrhosis. However, no correlation between serum growth hormone levels and the severity of glucose intolerance has been observed (Collins et al 1970, Leatherdale et al 1980) and insulin resistance has been demonstrated in a group of cirrhotics with normal serum growth hormone levels (West et al 1975). Although elevation of serum growth hormone within physiological limits may induce a degree of insulin resistance (Rizza et al 1983), such an effect cannot be assumed in cirrhotic subjects as the sensitivity to such a change may not be normal. Basal hyperglucagonaemia has frequently been reported in cirrhotic subjects (Sherwin et al 1978, Greco et al 1979, Dudley et al 1979, McDonald et al 1979) and immunoreactive glucagon has been shown to be the biologically active,

pancreatic form of glucagon (molecular weight 35,000) (McDonald et al 1979, Dudley et al 1979). The sensitivity of the plasma cyclic A.M.P. response to exogenous glucagon is normal in cirrhotic subjects (Strange et al 1977, Radziuk et al 1977). However the degree of hyperglucagonaemia has been reported to correlate with the degree of portosystemic shunting rather than hepatocellular function (Dudley et al 1979, Sherwin et al 1978) and no correlation of serum immunoreactive glucagon levels and glucose intolerance could be demonstrated (Riggio et al 1982). Morning serum cortisol levels have been reported to be normal in cirrhotic subjects (Riggio et al 1982, Johnston et al 1982) but the diurnal pattern is disturbed, evening serum free cortisol levels being elevated (Johnston et al 1982). It would appear unlikely that the counter-regulatory hormones, individually or in concert (Riggio et al 1982) could play a major role in the insulin resistance of chronic liver disease.

Circulating non-esterified fatty acid levels are raised in cirrhosis (Leatherdale et al 1980, Riggio et al 1982, Owen et al 1981) and this could decrease insulin mediated glucose utilisation by muscle (Randle et al 1963). Although a correlation between non-esterified fatty acid levels and glucose intolerance has been reported (Riggio et al 1982), the possibility that this is a secondary effect of the insulin resistance cannot be excluded.

The in vivo resistance to insulin action could be located either in the liver or in the peripheral tissues, muscle and fat. Direct study of the forearm tissues in cirrhotic man has demonstrated peripheral tissue resistance to insulin-mediated glucose uptake, similar rates of glucose uptake as in control subjects being observed despite higher serum insulin levels (Leatherdale et al 1980). If the insulin resistance represents a cellular defect rather than an effect of circulating factors, it could result from defects at any level from the initial event of hormone-receptor binding to the activation of the glucose transport system and the enzymes responsible for glucose metabolism. Isolated adipocytes were studied in order to localise any such defects to either receptor or post-binding steps of insulin action. In addition, the response to an oral glucose load was assessed and in vivo insulin sensitivity of glucose metabolism was

measured to allow comparison between the degree of *in vivo* abnormalities and any abnormality found at the cellular level.

SUBJECTS AND PROTOCOL

Sixteen patients with stable biopsy-proven cirrhosis who did not have clinical signs of porto-systemic shunting, ascites or encephalopathy were studied. No selection criteria concerning aetiology of cirrhosis were applied, and the group comprised six patients with alcholic cirrhosis, six patients with primary biliary cirrhosis (P.B.C.) and four patients with cryptogenic cirrhosis. All subjects were taking an adequate diet (minimum 200 grams of carbohydrate daily) and were ambulant. Two patients were receiving cholestyramine, two penicillamine, one cimetidine, four frusemide and four spironolactone. No drugs were taken on the morning of a test day. Eleven control subjects who had normal liver function tests, were taking no drugs and had no evidence of any metabolic disorder were also studied. The clinical and biochemical details of the patient and control group are given in Table 4.1 and those of each of the aetiological sub-groups of the cirrhotic group are given in Table 4.2.

Each subject was studied on three separate days. On the first day, blood was taken for monocyte studies and a glucose tolerance test was carried out. An adipose tissue biopsy was taken on the second day and *in vivo* insulin sensitivity was assessed on the third day. Insulin sensitivity was measured by the euglycaemic clamp technique in the first eight cirrhotic subjects and by the glucose/ insulin infusion technique in all subsequent subjects.

RESULTS

a) Glucose Tolerance Tests

Mean fasting blood glucose was similar in the cirrhotic and control group (4.2 $\pm$ 0.19 and 4.1 $\pm$ 0.14 mmol/l respectively) (Figure 4.1). After the glucose load, mean blood glucose levels were higher

in the cirrhotic group becoming significantly different from the control group within 45 minutes and remaining elevated thereafter (Figure 4.1). Nine of the group of 16 cirrhotics had impaired glucose tolerance as defined by the WHO criteria (WHO 1980). Mean fasting serum I.R.I. was higher in the cirrhotic group (12.1 ± 1.4 vs 7.0 ± 1.7 mU/l; $p < 0.01$) as was the peak I.R.I. (85.8 ± 7.5 vs 57.0 ± 10.5 mU/l; $p < 0.05$). Values were also significantly higher at 90, 120 and 150 min. Mean fasting serum proinsulin was grossly elevated in the cirrhotic group (43.2 ± 5.8 vs 17.2 ± 2.7 pmol/l) and remained elevated throughout the test (Figure 4.2). As proinsulin exhibits 50% cross-reactivity with insulin in the insulin radioimmunoassay used, a correction of the observed serum I.R.I. values was calculated for the effect of the presence of excess proinsulin. Although this reduced the magnitude of the difference between the cirrhotic and control groups (fasting: 9.0 ± 1.5 vs 5.7 ± 1.5 mU/l; peak: 71 ± 8.1 vs 49.0 ± 9.2 mU/l) (Figure 4.3) some cirrhotic patients remained markedly hyperinsulinaemic. Mean fasting C-peptide was higher in the cirrhotic group (0.51 ± 0.04 vs 0.34 ± 0.03 nmol/l; $p < 0.05$) but the C-peptide response to oral glucose appeared blunted and delayed. The mean incremental C-peptide responses did not differ significantly (0.86 ± 0.11 vs 1.02 ± 0.13 nmol/l respectively).

Mean fasting and peak blood glucose levels were similar in the alcoholic, P.B.C. and cryptogenic groups (Table 4.3). Mean peak blood glucose levels (9.8 ± 0.7, 10.3 ± 1.7 and 10.9 ± 0.8 mmol/l respectively; N.S.) were observed at 60 minutes in each group. Mean blood glucose fell more quickly in the P.B.C. group: 6.0 ± 0.8 mmol/l at 120 minutes compared with 9.2 ± 1.6 mmol/l for the alcoholic group ($p < 0.05$) and 8.9 ± 1.5 mmol/l for the cryptogenic group ($p = 0.057$). Mean fasting serum I.R.I. was lower in both the P.B.C. (10.0 ± 1.0 mU/l) and the cryptogenic group (7.9 ± 1.2 mU/l) than in the alcoholic group (16.4 ± 1.3 mU/l; $p < 0.05$) (Table 4.3).

b) Euglycaemic clamps

Euglycaemic hyperinsulinaemic clamps were performed on the first eight cirrhotic subjects studied (mean age 52.3 ± 5.3 years; 103 ± 8% ideal body weight; four male/four female) and on the first six control subjects studied (mean age 44.8 ± 0.2 years; 112 ± 5% I.B.W.;

three male/three female). The cirrhotic subjects demonstrated marked insulin resistance, the mean rate of glucose disposal being 3.4 ± 0.5 mg/kg/min compared with 8.3 ± 0.9 mg/kg/min for the controls ($p < 0.001$). The mean steady state serum I.R.I. was 45.4 ± 3.8 vs 40.3 ± 2.4 mU/l and mean steady state blood glucose was 4.4 ± 0.1 vs 4.3 ± 0.1 mmol/l for the cirrhotics and controls respectively. The mean fasting blood glucose levels were 4.5 ± 0.1 and 4.3 ± 0.1 mmol/l, and the coefficients of variation of clamp blood glucose were 3.1 ± 0.2% and 4.8 ± 0.3% respectively. Mean glucose disposal rates during the euglycaemic clamp were 4.4 ± 0.9 mg/kg/min for the P.B.C. group (n=4) and 2.5 ± 0.03 mg/kg/min for the alcoholic and cryptogenic subjects together ($n = 4$; $p = 0.09$).

c) Glucose/insulin infusions

The results of the glucose/insulin infusions which were performed on the last 9 cirrhotic subjects and 8 of the control subjects are shown in Figure 4.4. Mean steady state blood glucose levels were highly significantly different (8.3 ± 1.0 vs 2.7 ± 0.2 mmol/l at 120 min; $p < 0.001$). Steady state serum I.R.I. was markedly higher in the cirrhotic group than in the normal group (65.2 ± 6.5 vs 42.8 ± 3.8 mU/l at 120 minutes; $p < 0.05$). Insulin secretion was suppressed in the control group, C-peptide levels falling steadily over the last 90 minute of the infusion, but in the cirrhotic group C-peptide levels rose to a peak level at 120 minutes (1.14 ± 0.19 vs 0.32 ± 0.07 nmol/l, $p < 0.01$) (Figure 4.4). The P.B.C. group were less insulin resistant than either the alcoholic or the cryptogenic groups. Mean steady state blood glucose was 5.3 ± 0.73 mmol/l for the P.B.C. group and 10.6 ± 1.1 mmol/l for the combined alcoholic and cryptogenic groups ($p < 0.05$).

d) Blood intermediary metabolite levels

Fasting blood glycerol was 0.08 ± 0.01 mmol/l for the cirrhotic group and 0.04 ± 0.01 mmol/l for the control group ($p < 0.01$). There were no significant differences between the cirrhotic and control groups in the fasting levels of blood lactate (0.84 ± 0.09 vs 0.73 ± 0.07 mmol/l respectively) or 3-hydroxybutyrate (0.099 ± 0.023 vs 0.056 ± 0.024 mmol/l respectively). During the steady state period of the glucose/insulin infusions there were no differences between the

cirrhotic and control group in the levels of blood lactate (1.13 ± 0.11 vs 1.07 ± 0.09 mmol/l), 3-hydroxybutyrate (0.012 ± 0.003 vs 0.008 ± 0.001 mmol/l) nor glycerol (0.03 ± 0.01 vs 0.03 ± 0.01 mmol/l).

e) Monocyte insulin binding

There was no significant difference between the mean percentage insulin binding to monocytes from cirrhotic and control subjects (2.15 ± 0.16 vs 1.92 ± 0.31% per 10^6 monocytes respectively) (Figure 4.5). Half-maximum displacement of tracer was observed at 125 ± 13 and 132 ± 10 pmol/l respectively. There were no significant differences in maximum insulin binding between each of the cirrhotic sub-groups (2.02 ± 0.2%, 2.24 ± 0.33 and 1.90 ± 0.34% for alcoholic, P.B.C. and cryptogenic groups respectively).

f) Adipocyte insulin binding

The adipocyte insulin binding displacement curves are shown in Figure 4.6. The adipocytes from the cirrhotic patients bound significantly less insulin at tracer insulin concentrations (2.21 ± 0.12 vs 2.64 ± 0.13 % per 10 cm^2 cell membrane; $p < 0.05$). Half-maximum displacement of bound insulin occurred at 139 ± 11 pmol/l for the control group and 172 ± 17 pmol/l for the cirrhotic group ($p > 0.1$). Scatchard analysis suggested that the difference in maximum binding was largely a result of decreased receptor number on the adipocytes from cirrhotic patients (53.4 ± 4.9 vs 63.4 ± 4.8 receptors per μm^2 membrane) (Figure 4.6). Maximum binding to adipocytes from the P.B.C. and cryptogenic groups was similar (2.44 ± 0.17 and 2.40 ± 0.10 %). Adipocytes from the alcoholic group bound significantly less insulin (1.89 ± 0.20 % per 10 cm^2 cell membrane) than either the non-alcoholic cirrhotics ($p < 0.05$) or the normal subjects ($p < 0.001$). There was no significant difference between the insulin concentrations required for half-maximum displacement of tracer insulin for the alcoholic, P.B.C. and cryptogenic groups (178 ± 29, 143 ± 15 and 208 ± 45 pmol/l respectively).

g) In vitro lipogenesis

The basal rate of lipogenesis was not significantly different in adipocytes from cirrhotic and control subjects, but the mean maximum

rate of lipogenesis was greater for the cirrhotic group ($p < 0.05$) (Figure 4.7). However, Figure 4.8 shows that the cirrhotic patients do not form a homogenous group with respect to absolute rates of adipocyte lipogenesis. Adipocytes from the cryptogenic group achieved significantly higher rates of lipogenesis than those from the alcoholic group at all insulin concentrations and than those from the P.B.C. group at all insulin concentrations above 135 pmol/l. The P.B.C. group achieved significantly higher rates of lipogenesis than the alcoholic group at all insulin concentrations above 27 pmol/l. There were no significant differences between the control group and any sub-group in basal rates of lipogenesis, but both the P.B.C. and cryptogenic groups achieved higher maximal rates of lipogenesis ($p < 0.05$ and $p < 0.01$ respectively). Adipocytes from cirrhotic subjects were relatively resistant to insulin stimulation (half-maximum stimulation at 60.0 $\pm$ 8.0 pmol/l for cirrhotics and 21.8 $\pm$ 3.3 pmol/l for controls; $p < 0.001$). The half-maximal values for the alcoholic, P.B.C. and cryptogenic sub-groups were 46 $\pm$ 8, 66 $\pm$ 15 and 69 $\pm$ 19 pmol/l respectively ($p < 0.05$, $p < 0.01$ and $p < 0.01$ respectively compared with control group).

h) Relationship between cellular insulin binding and *in vivo* insulin sensitivity

There was a direct correlation between specific insulin binding to adipocytes and *in vivo* insulin sensitivity as determined by the glucose/insulin infusion technique both for the cirrhotic group ($R_s = 0.63$; $p < 0.05$) and for the cirrhotic and control subjects together ($R_s = 0.74$; $p < 0.01$) (Figure 4.9). Similarly there was a negative correlation between fasting serum I.R.I. and specific insulin binding to adipocytes (Figure 4.9) both for the cirrhotic group ($R_s = 0.54$; $p < 0.05$) and for the whole group ($R_s = 0.43$; $p < 0.05$). The cirrhotic group were relatively homogenous with respect to euglycaemic clamp data, and no correlation with adipocyte insulin binding could be demonstrated. Similarly, there was no correlation within the control group between the relatively narrow range of normal steady state blood glucose levels (1.8 to 3.5 mmol/l) and adipocyte specific insulin binding. Monocyte insulin binding correlated neither with *in vivo* insulin sensitivity nor with fasting serum I.R.I. (Figure 4.10).

	CIRRHOTICS n = 16	CONTROLS n = 11
Age (years)	53 ± 3	42 ± 4
Male/female ratio	9/7	6/5
% I.B.W.	108 ± 6	111 ± 4
Adipocyte diameter (μ)	102 ± 5	101 ± 6
Diet - grams carbohydrate/day	275 ± 26	261 ± 32
Diet - kcal/day	2217 ± 168	2400 ± 149
Bilirubin (μmol/l)	38 ± 12	8 ± 1
A.S.T. (IU/l)	81 ± 11	25 ± 6
Alkaline phosphatase (IU/l)	400 ± 86	61 ± 12
Albumin (grams/l)	36.7 ± 1.4	44.0 ± 1.2
Prothrombin ratio	1.1 ± 0.03	-

TABLE 4.1 Clinical and metabolic details of the cirrhotic and control groups.

	ALCOHOLIC n = 6	P.B.C. n = 6	CRYPTOGENIC n = 4
Age	49 ± 4	61 ± 5	49 ± 8
Male/female ratio	6/0	0/6	3/1
% I.B.W.	117 ± 7	90 ± 3	121 ± 15
Adipocyte diameter (μ)	114 ± 7	95 ± 7	96 ± 14
Diet - grams carbohydrate /day	291 ± 17	227 ± 35	318 ± 38
Diet - kcal/day	2329 ± 129	1995 ± 174	2480 ± 219
Bilirubin (μmol/l)	19± 5	*61 ± 31	32 ± 7
A.S.T. (IU/l)	74 ± 11	88 ± 22	80 ± 30
Alkaline phosphatase (IU/l)	160 ± 39	789 ± 99	179 ± 34
Albumin (grams/l)	39.2 ± 2.0	36.8 ± 1.9	32.8 ± 3.1
Prothrombin ratio	1.1 ± 0.8	1.0 ± 0.02	1.3 ± 0.03

TABLE 4.2 Clinical and metabolic features of the subgroups of cirrhotic subjects. *The P.B.C. group included the only jaundiced patient of this series (serum bilirubin 210 μmol/l).

	ALCOHOLIC	P.B.C.	CRYPTOGENIC	CONTROLS
GLUCOSE				
Fasting	#4.6±0.3	4.1±0.1	3.8±0.2	4.1±0.1
60 min	#9.8±0.7	#10.3±1.7	#10.9±0.8	*5.5±0.4
120 min	#9.2±1.6	#*6.0±0.8	#8.9±1.5	*3.9±0.4
INSULIN				
Fasting	#16.4±1.3	*10.0±1.01	*7.9±1.2	*7.0±1.7
60 min	#95.5±14.0	80.0±12.7	*52.5±9.4	*55.7±7.5
120 min	#84.8±13.2	#*42.2±9.6	#59.6±6.8	*18.2±2.9

TABLE 4.3 Blood glucose (mmol/l) and serum I.R.I. (mU/l) during oral glucose tolerance test in the cirrhotic subgroups.
* $p < 0.05$ compared with alcoholic group.
# $p < 0.05$ compared with control group.

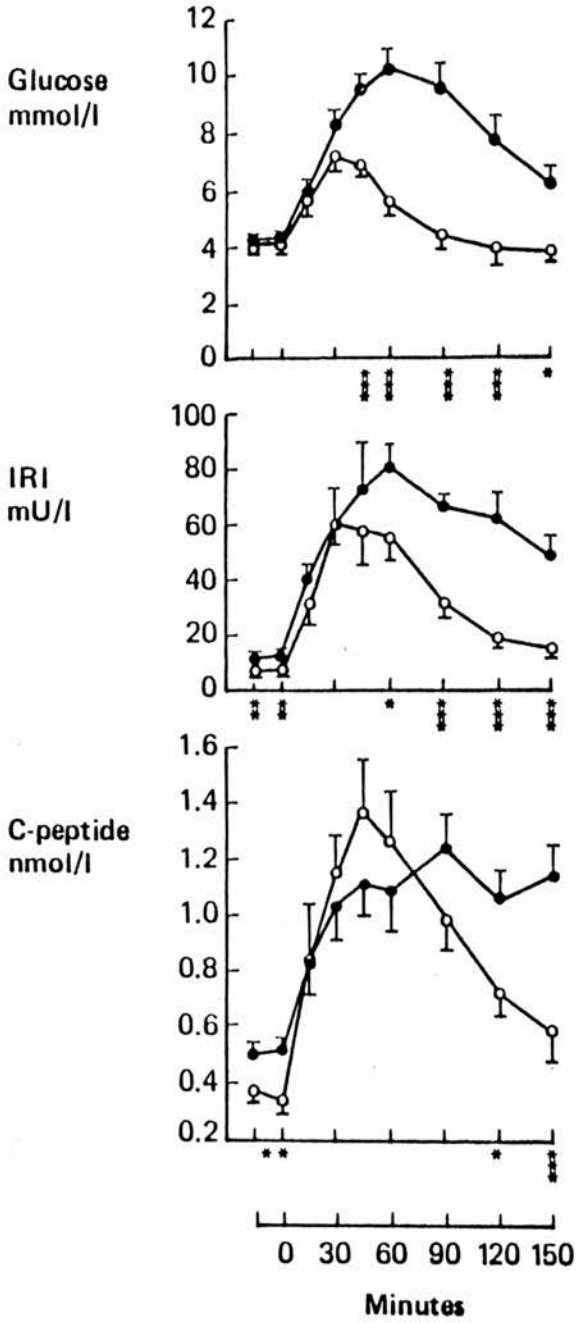

FIGURE 4.1 Mean blood glucose, serum I.R.I. and serum C-peptide response to 75 grams oral glucose.

Cirrhotic group (●——●), control group (o——o).

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$

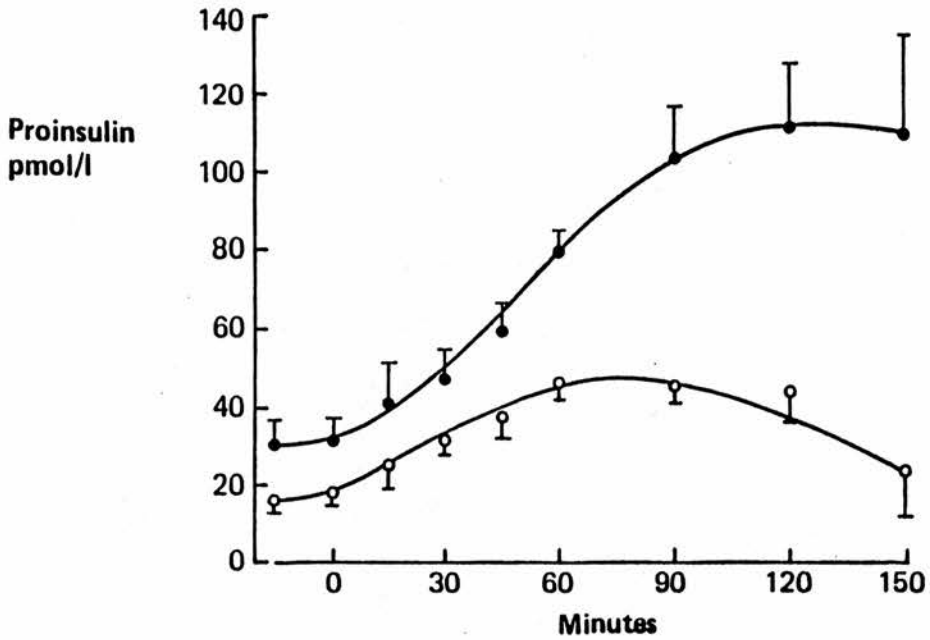

FIGURE 4.2 Mean serum proinsulin levels during oral glucose tolerance test.
Cirrhotic group (●——●), control group (o——o).
$p < 0.01$ at all time points.

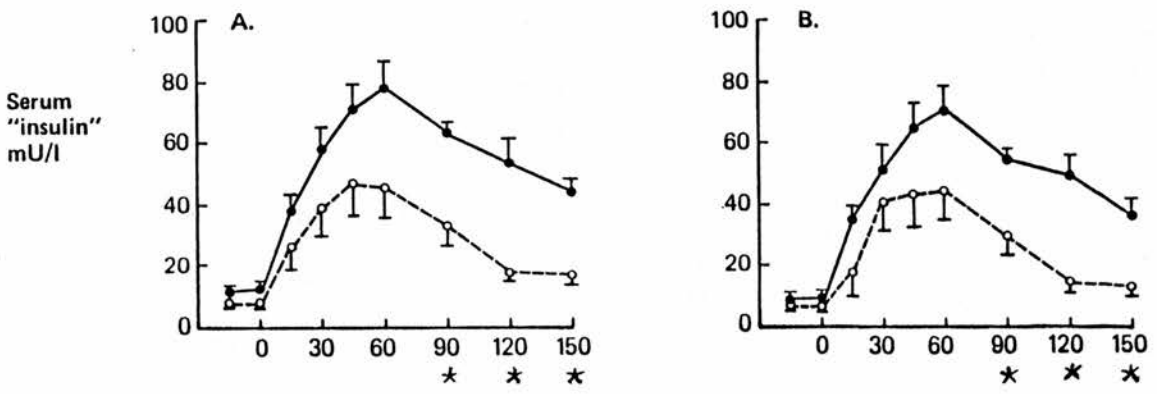

FIGURE 4.3 Mean serum immunoreactive insulin levels (panel A) and mean serum insulin levels corrected for proinsulin cross-reactivity (panel B).
Cirrhotic group n=16 (●——●), control group n=6 (o- -o).
* $p < 0.01$

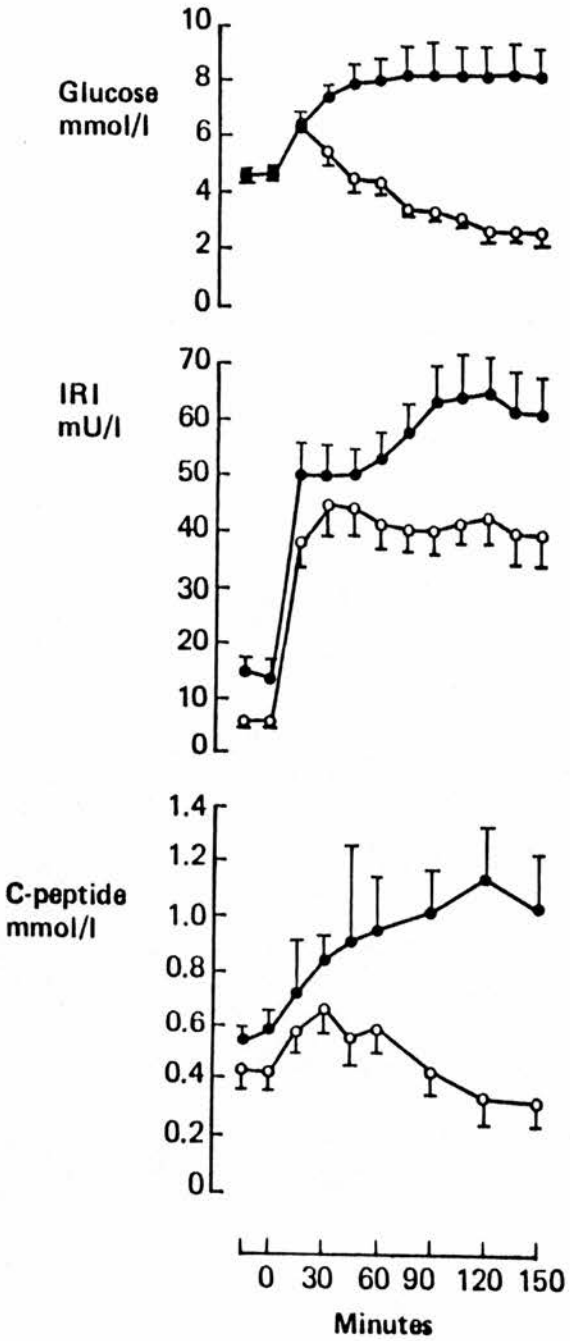

FIGURE 4.4 Mean blood glucose, serum I.R.I. and serum C-peptide changes during glucose/insulin infusion. Cirrhotic group n = 9 (●——●), control group n=8 (o——o)

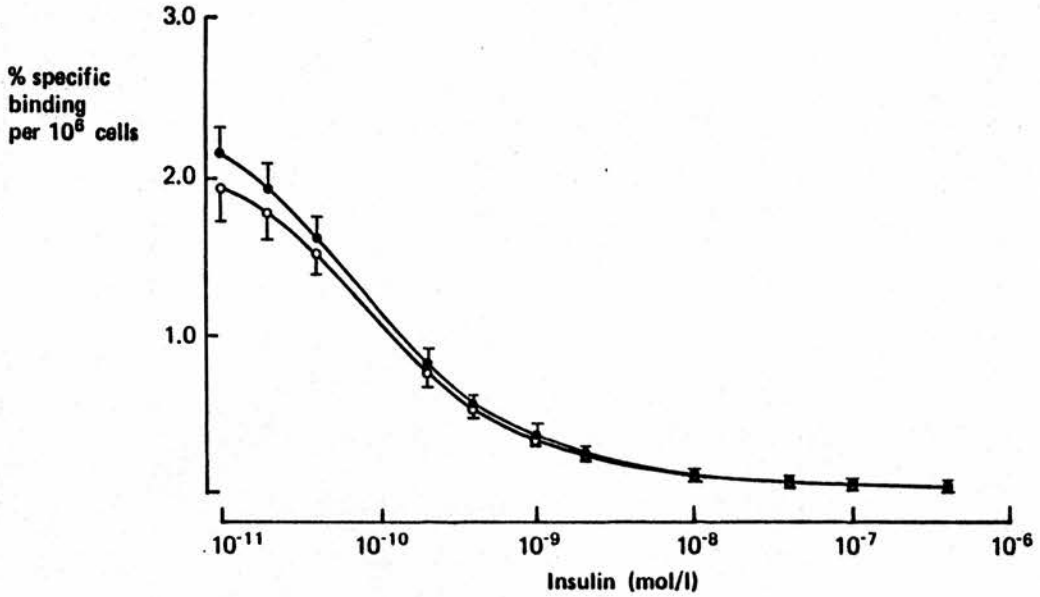

FIGURE 4.5 Specific insulin binding to monocytes.

Cirrhotic group n = 14 (●——●).

Control group n = 11 (o——o).

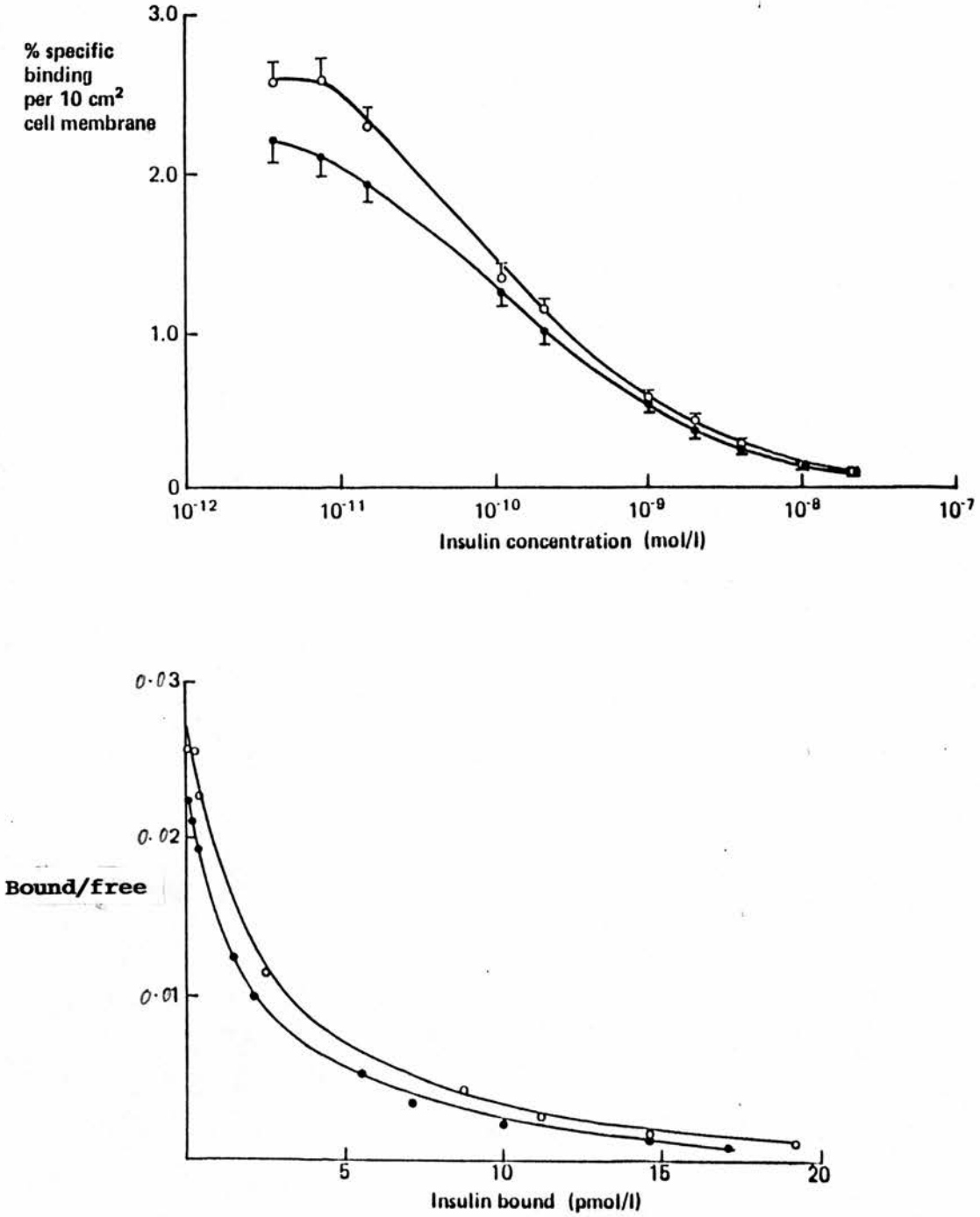

FIGURE 4.6 Specific insulin binding to adipocytes displayed as binding displacement curves (top) and Scatchard plots (bottom).

Cirrhotic group n = 16 (●——●).

Control group n = 11 (o——o).

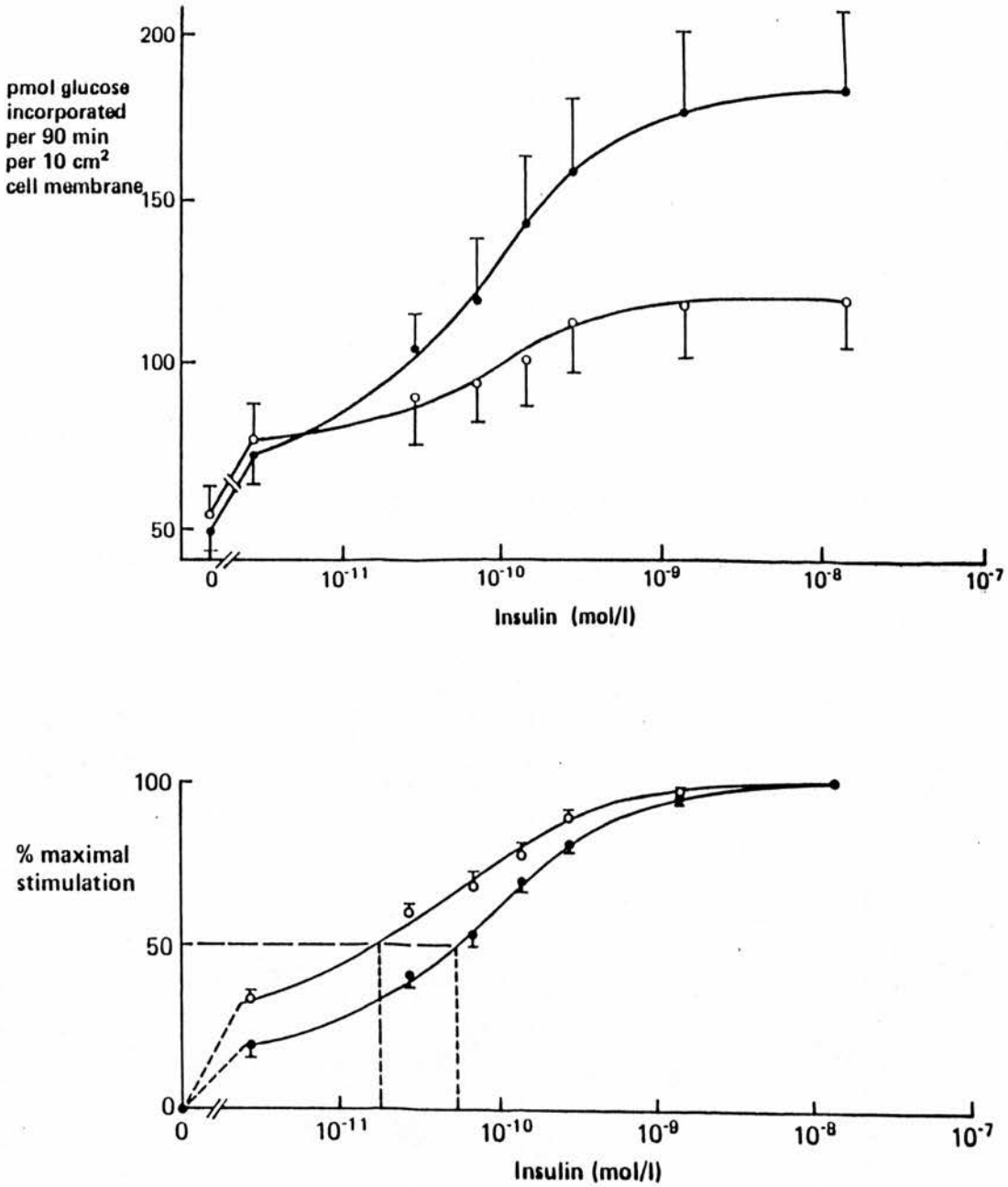

FIGURE 4.7 Insulin stimulation of lipogenesis in adipocytes shown as absolute rates and as percentage stimulation.

Cirrhotic group n = 16 (●——●).

Control group n = 11 (○——○).

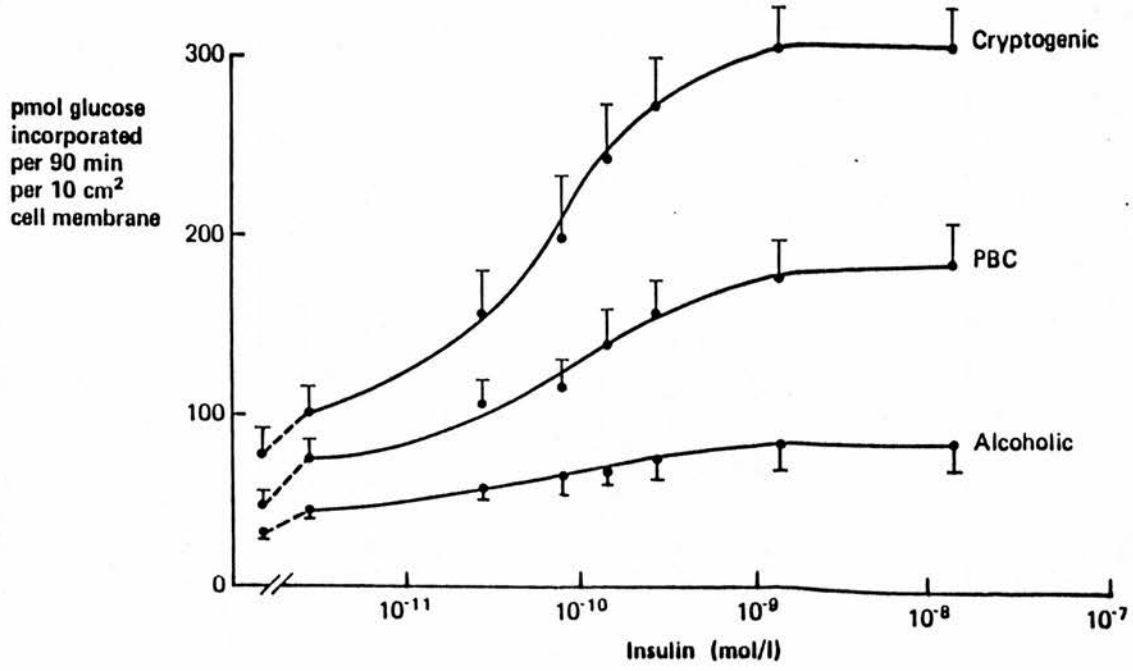

FIGURE 4.8 Insulin stimulation of lipogenesis in adipocytes from patients with cryptogenic (n = 4), primary biliary (n = 6) and alcoholic (n = 6) cirrhosis.

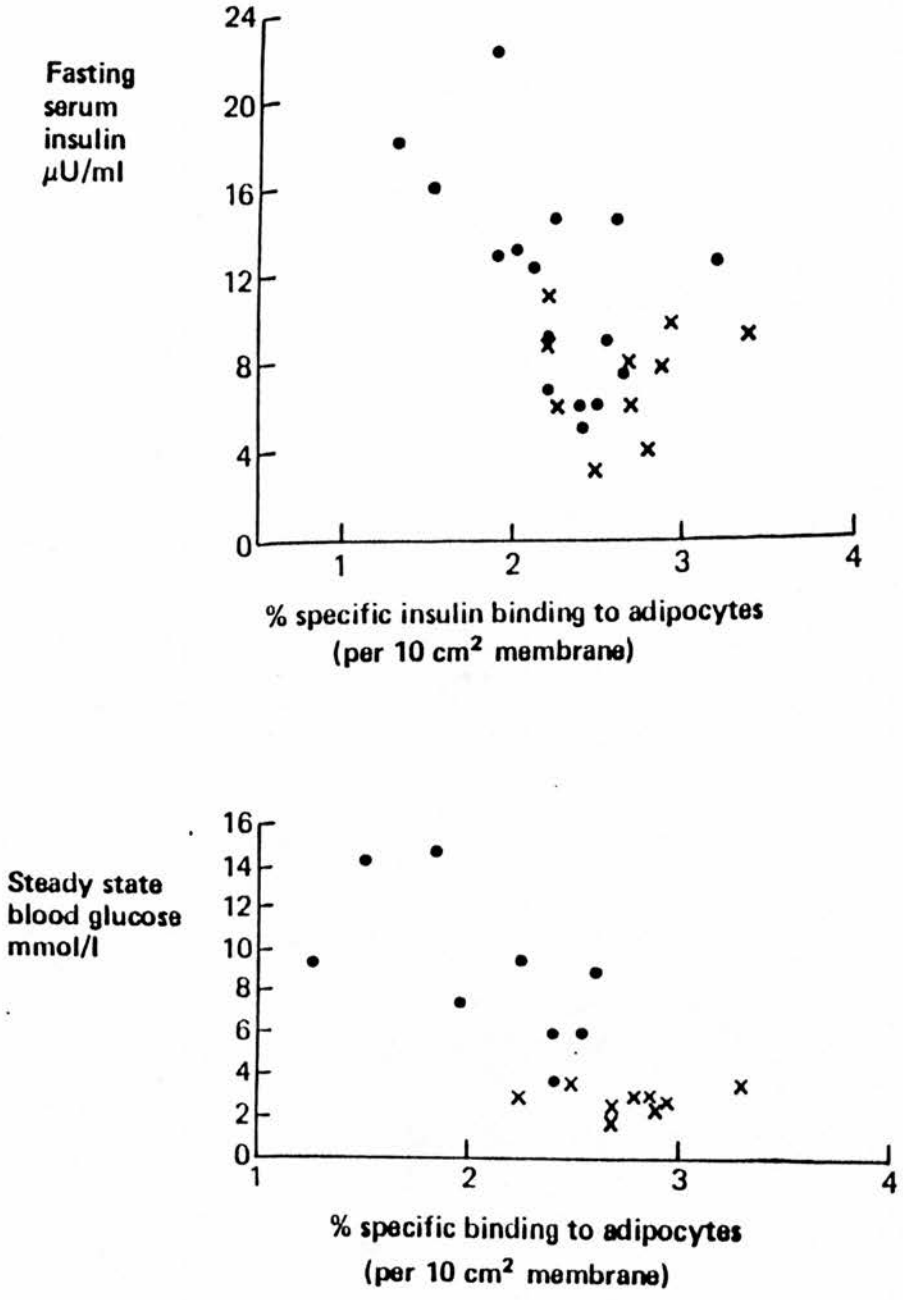

FIGURE 4.9 Relationship between adipocyte insulin binding and fasting serum insulin (top: R_s = 0.54, $p < 0.05$ for cirrhotics) and steady state blood glucose (bottom: R_s = 0.63, $p < 0.05$ for cirrhotics).

Cirrhotic (•) and control (x) subjects.

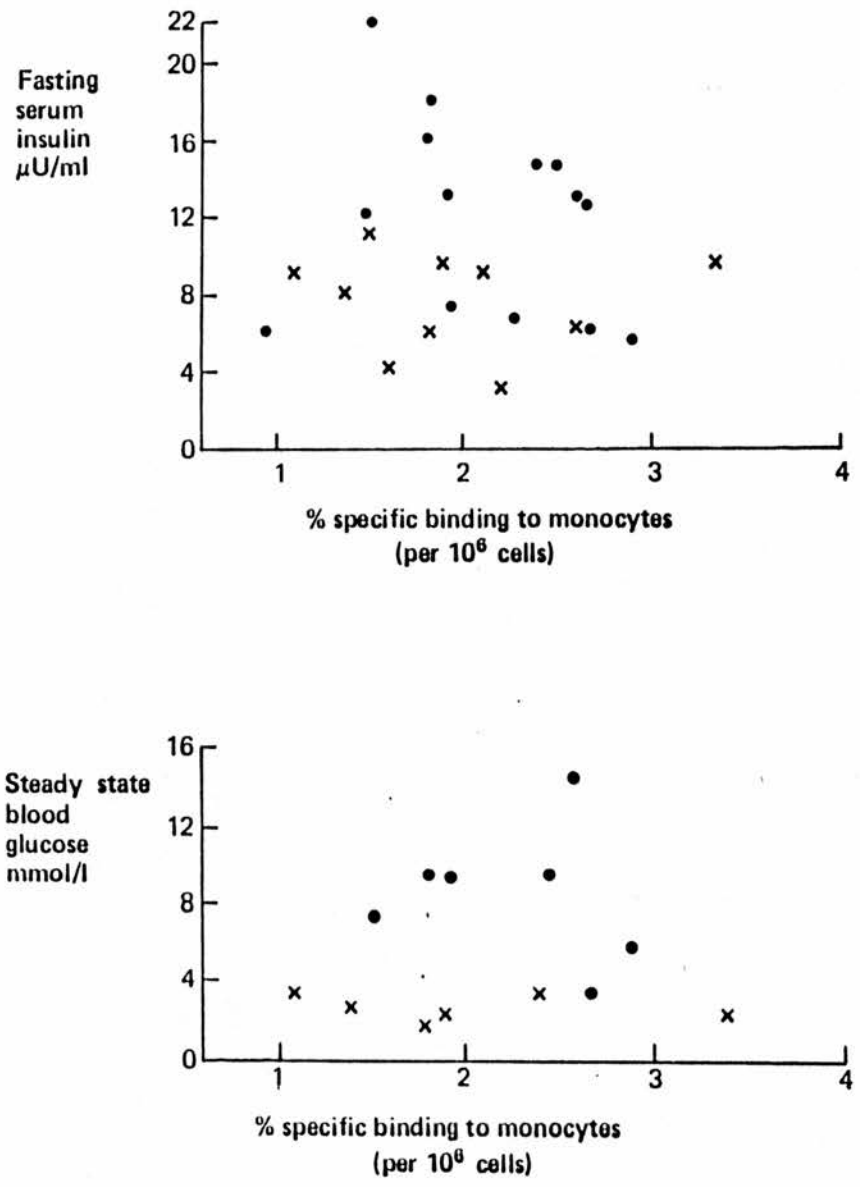

FIGURE 4.10 Relationship between monocyte insulin binding and fasting serum insulin (top: R_s = 0.32 for cirrhotics) and steady state blood glucose (bottom: R_s = 0.63 for cirrhotics).

Cirrhotic (•) and control (x) subjects.

DISCUSSION

The oral glucose tolerance tests in the cirrhotic subjects demonstrated impairment of glucose tolerance, fasting hyperinsulinaemia and delayed, exaggerated peak insulin responses which have been previously demonstrated (Collins et al 1980, Riggio et al 1982, Johnston et al 1977). The cause of the hyperinsulinaemia has been reported to be a result of impaired insulin degradation by some workers (Riggio et al 1982) and hypersecretion of insulin by others (Megyesi et al 1967, Johnston et al 1977). Our observation of raised fasting C-peptide levels, but lesser change in serum C-peptide after oral glucose in the cirrhotic subjects would suggest that the fasting hyperinsulinaemia was secondary to an increased basal insulin secretion rate, but that impaired hepatic extraction or degradation allowed post-prandial hyperinsulinaemia. Any such impaired insulin handling could be a result of porto-systemic shunting or intrinsic hepatic abnormalities. As post-prandial hyperinsulinaemia is not observed in patients with portal vein thrombosis and normal liver function (Johnston et al 1978), the latter possibilty appears to be more likely. Previous studies have attempted to explain the abnormalities of insulin secretion in cirrhosis on the basis of a single defect (Megyesi et al 1967, Johnston et al 1977, Riggio et al 1982). The possibility has not been considered to date of both basal hypersecretion and impaired hepatic degradation, the relative contributions of each to overall serum insulin levels varying with insulin secretion rate. The abnormal temporal pattern of C-peptide and therefore of insulin secretion must be considered in relation to possible abnormal absorption of the glucose load in cirrhotic subjects.

A further point in the consideration of hyperinsulinaemia is the crossreactivity of insulin and proinsulin in the radioimmunoassays. Previously it has been assumed that I.R.I. directly reflected biologically active insulin in cirrhotic subjects. Correction for this artefact of measurement (Figure 4.3) reveals that although hyperinsulinaemia is present in some patients, the degree of abnormality is less than has been reported. Recently, hyperproinsulinaemia in cirrhosis has been confirmed by a group using

a different proinsulin assay (Kasperska-Czyzyzkowa et al 1983). The hyperproinsulinaemia could result from diminished degradation of proinsulin, but at least 50 % of proinsulin is cleared via the kidneys (Katz & Rubenstein 1973) and abnormal secretion of proinsulin cannot be excluded at present.

Nine of the group of 16 cirrhotic subjects had impaired glucose tolerance as defined by the WHO criteria (1980). The degree of impairment of glucose tolerance bore no relationship to adipocyte insulin binding or insulin sensitivity nor to measured insulin sensitivity in vivo. The latter assessment relates almost entirely to the peripheral tissues (DeFronzo et al 1983). It is thus likely that peripheral tissue insulin resistance is but one factor involved in the pathogenesis of impaired glucose tolerance. As approximately 50% of an oral glucose load is taken up by the liver (Waldhausl et al 1983) it is apparent that reduction of the functional parenchymal mass or a reduction in sensitivity to insulin stimulated glucose metabolism or storage may be very significant in the pathogenesis of impaired glucose tolerance in cirrhosis.

The existence of resistance to insulin action in cirrhosis has long been postulated on the basis of indirect studies (Collins et al 1970, Johnston et al 1977). Human forearm studies showed that glucose uptake was normal in the presence of hyperinsulinaemia, suggesting peripheral tissue insulin resistance (Leatherdale et al 1980). Recent studies using the euglycaemic hyperinsulinaemic clamp technique have reported marked peripheral insulin resistance in cirrhosis (Iversen et al 1984, Vannini et al 1984). Similar observations were made in the present study. The low rates of glucose disposal recorded in the cirrhotic subjects did not allow confident ranking of the subjects with respect to insulin resistance. In order to achieve better separation between the more severe degrees of insulin resistance, the glucose/insulin infusion technique (Harano et al 1978, Heine et al 1982) was used in the second part of the study. The marked resistance to the action of insulin in promoting glucose disposal was fully displayed using this technique, and the apparent effect of the hyperglycaemia of the cirrhotic subjects in stimulating a degree of endogenous insulin secretion did not mask the insulin

resistance. Both the euglycaemic clamp and the glucose/insulin infusion techniques measure predominantly peripheral tissue glucose disposal, hepatic glucose production being effectively suppressed at the insulin infusion rate used (50 mU/kg/h) (DeFronzo *et al* 1983). Hepatic glucose output was not assessed in this study, and it must be considered that the peripheral tissue glucose disposal in the cirrhotic subjects might have been underestimated if hepatic glucose output was not adequately suppressed. However, even if hepatic glucose output was only 50% suppressed this would only increase the overall glucose disposal rate by approximately 1 mg/kg/min. Assessment of basal hepatic glucose output by both catheterisation and isotope techniques has suggested decreased hepatic glucose release in cirrhosis (Owen *et al* 1976, Perez *et al* 1978). It would thus appear unlikely that the *in vivo* insulin sensitivity in cirrhosis has been seriously underestimated in the present study. The finding of marked peripheral insulin resistance corroborates the *in vivo* observation on forearm glucose metabolism (Leatherdale *et al* 1980) but leaves open the question of its pathophysiological basis.

Adipocyte insulin binding was found to be reduced in the cirrhotic group, and the reduction correlated both with the elevation of fasting serum I.R.I. levels and the decreased *in vivo* insulin sensitivity. In the only other study of adipocyte insulin binding in cirrhosis, no significant decrease in binding was observed (Harewood *et al* 1982). In that study, adipocyte binding was performed on only six subjects with cirrhosis of unstated aetiology. The binding data were expressed per cell number and this could have resulted in underestimation of the true insulin binding in the cirrhotic group as the cells of the cirrhotics (predominantly male) were significantly smaller than those of the controls (predominantly female). The assay used by Harewood and colleagues was associated with non-specific binding of insulin of 27.7%, and this analytical difficulty may have contributed to the large standard errors of each group mean. Finally, the subjects studied were all undergoing porto-systemic shunt operations. In contrast, all the patients reported in the present study had no clinical evidence of portal hypertension and were in a stable condition.

Several previous studies have examined insulin binding to non-target organ cells in cirrhosis. In a group of alcoholic cirrhotics, monocyte insulin binding was found to be reduced (Greco et al 1980). Blei and co-workers observed no reduction in monocyte insulin binding in a group of 16 patients with cirrhosis of varying aetiology but found that the five hyperinsulinaemic cirrhotics had reduced monocyte insulin binding (Blei et al 1982). In a group of Japanese patients with unspecified liver disease, monocyte insulin binding was found to be reduced in direct proportion to the severity of impairment of glucose tolerance (Kanatsuna et al 1981), whereas in a large group of caucasian cirrhotics, monocyte insulin binding was normal in those subjects with impaired glucose tolerance and was reduced only in those subjects with normal glucose tolerance (Piniewski et al 1984). These conflicting results may reflect alterations in mononuclear cell subpopulations in different types of cirrhosis, or indeed any alterations in the monocyte staining properties as this would directly affect the results derived from the study of mixed mononuclear cell preparations. Lack of correlation between monocyte and adipocyte binding has previously been reported in normal subjects (Olefsky 1976) and insulin dependent diabetics (Pedersen & Hjollund 1982). The possible pitfalls in deriving physiological significance from results of monocyte binding studies necessarily performed at 20° C and pH 8.0 are evident, and these could account at least in part for the lack of correlation with the adipocyte binding studies as discussed in Chapter 3.

The finding of reduced adipocyte insulin receptor number is of interest, but the relationship of the hyperinsulinaemia to receptor downregulation and to the insulin resistance is unclear. It is possible that the receptor downregulation could be a secondary effect of the hyperinsulinaemia, the latter being both a response to post-receptor insulin resistance and a manifestation of impaired insulin degradation. The degree of resistance to adipocyte insulin stimulation of lipogenesis did not differ significantly between the three cirrhotic sub-groups although the adipocytes from the alcoholic subjects required a slightly lower mean insulin concentration for half-maximum stimulation. However, only the alcoholic sub-group displayed marked reduction of insulin binding to adipocytes. The

observed cellular insulin resistance did not relate to insulin receptor status. The decrease in sensitivity is in agreement with the data of Harewood et al (1982) which also reflected the greater maximum capacity of adipocytes from cirrhotic subjects to synthesise lipid in vitro. Caro and Amatruda (1980) have demonstrated that the process of downregulation involves post-binding pathways. It may be argued that the adipocytes from the non-alcoholic cirrhotic subjects had a greater post-binding defect and thus displayed slightly greater insulin resistance and failure to downregulate. Interpretation of the insulin sensitivity data is complicated by the different degree of reponsiveness of each group. Although it has been postulated that cellular hormone sensitivity (as represented by hormone concentration required for half-maximal stimulation) and cellular responsiveness (as represented by the increment over the basal metabolic state achieved by maximum hormone stimulation) are separate and independent parameters (Kahn 1978), uncertainty must exist when comparing the sensitivity of different cell populations with disparate responsiveness. Reduction in responsiveness is conventionally taken to indicate the presence of a post-binding defect and reduction in sensitivity to indicate the presence of a binding defect. These theoretical distinctions are not absolute (Kahn 1978). At the present time, the adipocyte insulin receptor downregulation of the alcoholic group may be more simply accounted for by the more marked hyperinsulinaemia in this group. An intracellular defect of insulin action appeared to be prominent in the sub-group with the least in vivo insulin resistance (P.B.C.), the in vitro adipocyte insulin resistance being associated with near normal insulin binding. Evidence for an important in vivo post-binding defect is provided by a dose response euglycaemic clamp study which demonstrated 30% reduced maximal capacity and 50% reduced insulin sensitvity to insulin stimulated glucose disposal in cirrhotic subjects (Iversen et al 1984).

The observation that the maximum capacity for lipogenesis differed between the three aetiological sub-groups reported here is surprising. It has previously been assumed that the metabolic effects of the cirrhosis would overshadow any differences associated with different aetiologies. Interpretation of this observation is

complicated by the lack of matching for sex and percentage ideal body weight between the sub-groups. The P.B.C. group were all female, were significantly thinner and had smaller adipocytes than the alcoholic or cryptogenic groups. A large series of studies upon normal males and females showed significantly higher rates of lipogenesis in females, whether the results were expressed per cell surface area or per cell number (Pedersen et al 1982a), but this observation does not explain the findings in the cryptogenic group. Severity of cirrhosis, as judged from clinical features, standard liver function tests and histological picture, did not differ between the groups although it is possible that hepatocyte function is better preserved in P.B.C. for an equivalent elevation of serum bilirubin and transaminase (Vierling 1982). The effect of alcohol ingestion upon maximal capacity for lipogenesis is not known, but as the subjects had been observed for at least 24 hours before biopsy, any such effect could not be a result of acute toxicity of alcohol, but may represent a chronic effect of the drug upon adipocytes.

In conclusion, this study has revealed important differences in adipocyte metabolism between the aetiological sub-groups of cirrhosis examined, despite the apparent similarity in severity of cirrhosis. All three sub-groups exhibited significant in vivo insulin resistance, but the patients with P.B.C. were less insulin resistant than those with alcoholic and cryptogenic cirrhosis. The alcoholic cirrhosis patients showed the greater reduction in adipocyte insulin binding and a sub-normal maximal stimulation of lipogenesis. The adipocytes from patients with cryptogenic cirrhosis differed from those of the alcoholic group mainly in achieving much higher rates of lipogenesis in response to insulin stimulation. The effect of chronic exposure to alcohol appears striking, but the precise cause of the differences between the groups must await further studies, not least because of the lack of matching of patient characteristics between the sub-groups and the control group. All the cirrhotic subjects studied displayed a major defect in adipocyte insulin sensitivity and this was reflected in the severe in vivo insulin resistance of this condition.

CHAPTER FIVE

CHRONIC RENAL FAILURE

INTRODUCTION

The occurrence of impaired glucose tolerance in chronic renal failure has been recognised for over 70 years (Neubauer 1910). The possibility of inadequate insulin secretion was debated until the advent of radioimmunoassay of serum insulin, whereafter elevated serum I.R.I. responses to oral glucose were uniformly observed (Hampers et al 1968, Spitz et al 1970, DeFronzo et al 1978a). The combination of impaired glucose tolerance and elevated serum I.R.I. suggested that insulin resistance was a feature of chronic renal failure. Insulin resistance in vivo could be a result of defects in insulin action in the liver, in the peripheral tissues or at both sites. The question of decreased sensitivity of peripheral tissues to insulin was approached directly by Westervelt (1969) who demonstrated that glucose uptake by the forearm muscle of uraemic patients was poorly stimulated by an exogenous insulin infusion. Spitz and colleagues (1970) noted decreased effectiveness of intravenous insulin in decreasing blood glucose and serum phosphate levels and concluded that muscle was insensitive to insulin in chronic renal failure. At the insulin concentrations used in euglycaemic clamp studies which also demonstrated insulin resistance (DeFronzo et al 1981b, Smith & DeFronzo 1982) it is likely that the insulin sensitivity of muscle and fat, rather than liver, was examined (DeFronzo et al 1983a). Hepatic glucose output in uraemic subjects suppressed normally at high physiological levels (Smith & DeFronzo 1982), but the possibility remains of hepatic insulin resistance at basal or low serum insulin levels. In uraemic rats, hepatic insulin resistance has been observed (Kauffman & Caro 1983). Hence, a defect in peripheral insulin sensitivity is established and the degree of hepatic insulin sensitivity is uncertain.

The cellular mechanism of insensitivity to insulin in chronic renal failure is uncertain. Evidence for the presence of circulating inhibitors of insulin action was put forward first by Westervelt and Schreiner (1962) and at intervals since (Glaze et al 1967, Dzurik et al 1969, Milutinovic et al 1983, Lockwood & McCaleb 1983). The nature of any such substance and its mode of action remains obscure. Elevations of growth hormone, cortisol, parathyroid hormone and

glucagon do not correlate with the degree of insulin resistance or glucose intolerance (Spitz et al 1970, DeFronzo et al 1983b). Studies of insulin receptors on circulating blood cells have yielded conflicting results, decreased receptor number on erythrocytes (Gambhir et al 1980 & 1981a) and normal receptor number on monocytes (Smith & DeFronzo 1982) having been observed. Resistance to insulin action at an intracellular, post-binding site was suggested by the finding of decreased insulin mediated glucose disposal in vivo even at serum insulin concentrations sufficient to achieve maximal insulin receptor occupancy (Smith & DeFronzo 1982).

The effects of dialysis treatment upon abnormalities of carbohydrate metabolism have been assessed. Uraemic patients on maintenance haemodialysis show improved glucose tolerance without a change in the serum I.R.I. response (Hampers et al 1968) and insulin-dependent diabetic patients on treatment require less insulin both basally and post-prandially (Schmitz et al 1984). These findings suggest an increase in insulin sensitivity. Direct measurement of insulin sensitivity using the euglycaemic clamp technique confirmed this hypothesis (DeFronzo et al 1978a). However, neither glucose tolerance nor tissue insulin sensitivity return to normal during chronic haemodialysis. Continuous ambulatory peritoneal dialysis (C.A.P.D.) is associated with a more normal life-style and better general health (Ramos et al 1983). A strict diet is not necessary during this form of dialysis and an adequate carbohydrate intake is assured, 80 to 250 grams of glucose per day being absorbed from the dialysate via the peritoneal cavity (Heaton et al 1983). Total energy intake on C.A.P.D. does not rise markedly as dietary intake tends to be spontaneously reduced (Heaton et al 1984). The impact of C.A.P.D. upon the tissue insulin sensitivity in chronic renal failure is unknown. In order to study the cellular changes associated with renal failure and the effect of C.A.P.D., adipocyte insulin binding, adipocyte insulin sensitivity and glucose tolerance were examined in a group of patients with advanced renal failure before and after three months of treatment.

SUBJECTS AND PROTOCOL

Nine subjects with chronic renal failure were recruited to the study. The selection criteria required that the subjects were 20 to 65 years of age, taking an adequate diet, generally fit and not receiving steroid therapy. None had a family history of diabetes. All subjects were about to commence C.A.P.D. therapy as renal function was steadily deteriorating. Each subject underwent a fat biopsy and a 75 gram oral glucose tolerance test. Six subjects were asked to participate in a repeat study after three months on C.A.P.D. Five were successfully restudied, the sixth patient suffering a fatal myocardial infarction before the tests were due to be repeated. In order to avoid significant glucose absorption from the peritoneal cavity over the ten hours prior to investigation, subjects on C.A.P.D. omitted their usual 2200 h peritoneal fluid exchange on the evenings before tests. The uraemic group were compared to an age and sex matched group of healthy subjects. Clinical and metabolic data on the uraemic group, the uraemic subgroup restudied on C.A.P.D. and the normal control group are given in Table 5.1.

RESULTS

a) Oral glucose tolerance test

Fasting blood glucose levels were significantly elevated in the uraemic group compared with the control group (5.0 $\pm$ 0.2 vs 4.1 $\pm$ 0.1 mmol/l respectively; $p < 0.001$) and remained so throughout the test (Figure 5.1). Maximum blood glucose levels were achieved after 60 minutes in the uraemic group and after 30 minutes in the control group. Seven of the nine uraemic subjects displayed impaired glucose tolerance (W.H.O. 1982) but two subjects did not rise above 8.5 mmol/l and fell to 4.8 and 2.1 mmol/l respectively at 120 minutes after the glucose load. Serum I.R.I. levels in the uraemic group were not significantly elevated in the fasting state (9.7 $\pm$ 1.3 vs 6.8 $\pm$ 0.9 mU/l). The maximum incremental rises were not significantly different in the uraemic group despite the continuing stimulus of hyperglycaemia, but serum I.R.I. remained significantly elevated after 90 minutes (Figure 5.1). Elevation of fasting serum proinsulin

(30.6 ± 6.0 vs 17.2 ± 2.7 pmol/l) accounted entirely for the slight increase in fasting serum I.R.I. in the uraemic group. Peak serum proinsulin levels were delayed in the uraemic group, but correction of I.R.I. data for proinsulin crossreactivity did not obviate the apparent hyperinsulinaemia after 90 minutes (Figure 5.2). Fasting C-peptide levels in the uraemic group were markedly elevated (1.13 ± 0.13 vs 0.34 ± 0.03 nmol/l; $p < 0.001$) and rose continuously throughout the test (Figure 5.1).

In the five subjects restudied during C.A.P.D., fasting blood glucose levels were found to have returned towards normal compared with before dialysis (4.5 ± 0.1 vs 5.3 ± 0.3 mmol/l; $p < 0.001$). The shape of the blood glucose profiles remained very similar, but blood glucose levels were consistantly lower throughout the test (Figure 5.3). Serum I.R.I. levels were almost identical before and during C.A.P.D. both fasting (9.7 ± 1.3 vs 9.6 ± 0.7 mU/l) and after glucose ingestion (51.6 ± 5.0 vs 51.0 ± 3.1 mU/l at 60 minutes; Figure 5.3). Serum proinsulin levels both fasting and during the glucose tolerance test were not changed by C.A.P.D. Fasting C-peptide levels appeared slightly higher during C.A.P.D. (1.14 ± 0.11 vs 1.76 ± 0.43 nmol/l) and rose more rapidly in the C.A.P.D. group, becoming significantly different from the pre-dialysis group at 30 and 45 minutes after the glucose load (Figure 5.3).

b) Adipocyte insulin binding

Maximum specific insulin binding was 2.55 ± 0.23 vs 2.57 ± 0.09 % per 10 cm^2 cell membrane in the uraemic and control groups respectively (Figure 5.4). The insulin concentrations at which half-maximum binding was observed were 91 ± 8 pmol/l (uraemic) and 139 ± 11 pmol/l (control) ($p < 0.005$). This indication of raised receptor affinity in the uraemic group was borne out by Scatchard analysis (Figure 5.4). The slopes of the high affinity portion of the curves were −0.021 ± 0.003 and −0.010 ± 0.001 for uraemic and control groups respectively ($p < 0.001$) and those of the low affinity portion of the curves were −2.85 ± 0.52 vs −1.95 ± 0.21 (not significant). The number of high and low affinity insulin receptors was smaller in the uraemic group (3.9 ± 0.4 vs 7.5 ± 0.6; $p<0.001$ and 44.5 ± 4.1 vs 63.3 ± 4.9 per μ^2 cell membrane; $p<0.01$).

Maximum specific insulin binding fell in all subjects during C.A.P.D. (2.95 ± 0.23 vs 2.12 ± 0.19 % per 10 cm^2 cell membrane; $p < 0.05$) (Figure 5.5). Half-maximum binding was observed at 86 ± 14 and 98 ± 10 pmol/l (N.S.). Scatchard analysis suggested that the observed change in maximum specific binding was a result of modest changes in both affinity and number of high affinity receptors (slope -0.025 ± 0.004 vs -0.020 ± 0.003 and receptor number (3.7 ± 0.7 vs 3.2 ± 0.5 per μm^2).

c) Adipocyte insulin sensitivity

Basal and maximal rates of lipogenesis were similar in the uraemic and control groups (47.5 ± 6.5 vs 54.6 ± 8.4 and 120 ± 24 vs 122 ± 15 pmol per 10 cm^2 cell membrane per 90 minutes respectively). Half-maximal stimulation of lipogenesis was observed at 13.5 ± 4.4 and 21.4 ± 3.0 pmol/l respectively (N.S.) (Figure 5.6).

During C.A.P.D., basal rates of lipogenesis were unchanged but maximally stimulated rates of lipogenesis appeared lower (116 ± 38 vs 82 ± 19 pmol per 10 cm^2 per 90 minutes; N.S.).The dose response curve was slightly shifted to the right during C.A.P.D. (half-maximal stimulation at 13.9 ± 4.7 vs 29.0 ± 13.0 pmol/l; N.S.) (Figure 5.7).

	URAEMIC SUBJECTS n=9	CONTROL SUBJECTS n=12	URAEMIC PRE-CAPD n=5	URAEMIC POST-CAPD n=5
Age (years)	45.6±5.1	42.1±3.5	48.6±7.7	49.1±7.7
Male/female ratio	5/4	6/6	2/3	3/2
% I.B.W.	105±3	114±4	109±5	110±5
Duration of uraemia (years)	4.2±1.2	-	2.4±0.2	2.8±0.2
Diet - energy (kcal/day)	2059±216	2360±126	1939±353	2223±297
- carbohydrate (g/day)	238±30	241±24	204±38	319±38
Adipocyte diameter (μ)	90±6	102±5	96±10	101±12
Serum creatinine (μmol/l)	1010±61	77±4	981±82	784±84
Serum bicarbonate (mmol/l)	16.2±1.1	25.1±0.4	17.4±1.7	23.0±0.3
Serum P.T.H. (U/l)	2.6±0.3	-	2.8±0.5	1.3±0.4

TABLE 5.1 Clinical and metabolic characteristics of the subjects with chronic renal failure, the normal control group and the subgroups of uraemic subjects studied both before and during C.A.P.D.

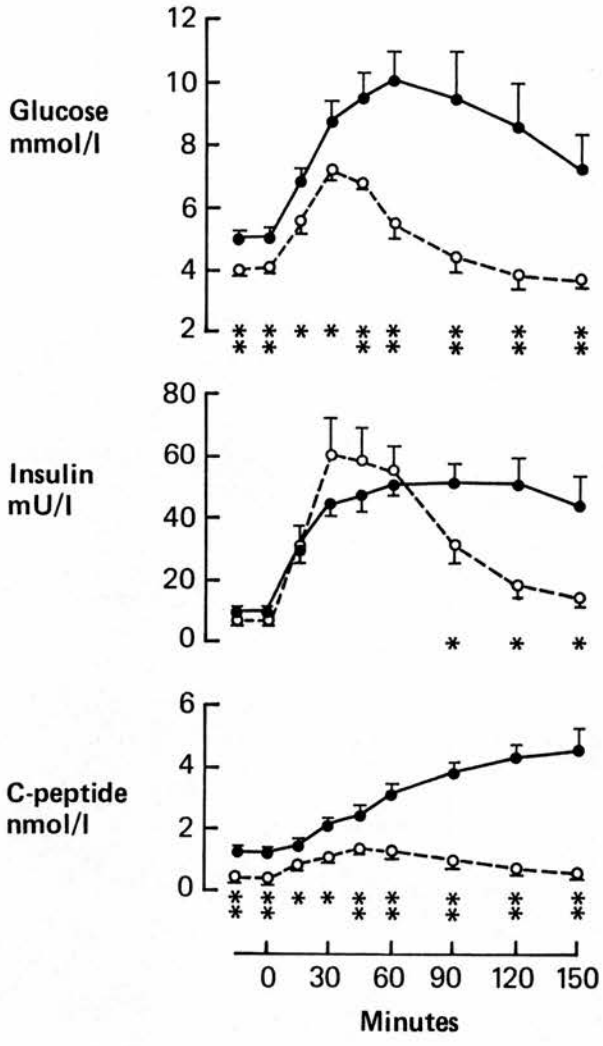

FIGURE 5.1 Mean blood glucose, serum I.R.I. and serum C-peptide responses to a 75 gram oral glucose load.

Uraemic n = 9 (●——●) and control n = 12 (o---o) groups.

* $p < 0.01$ ** $p < 0.001$

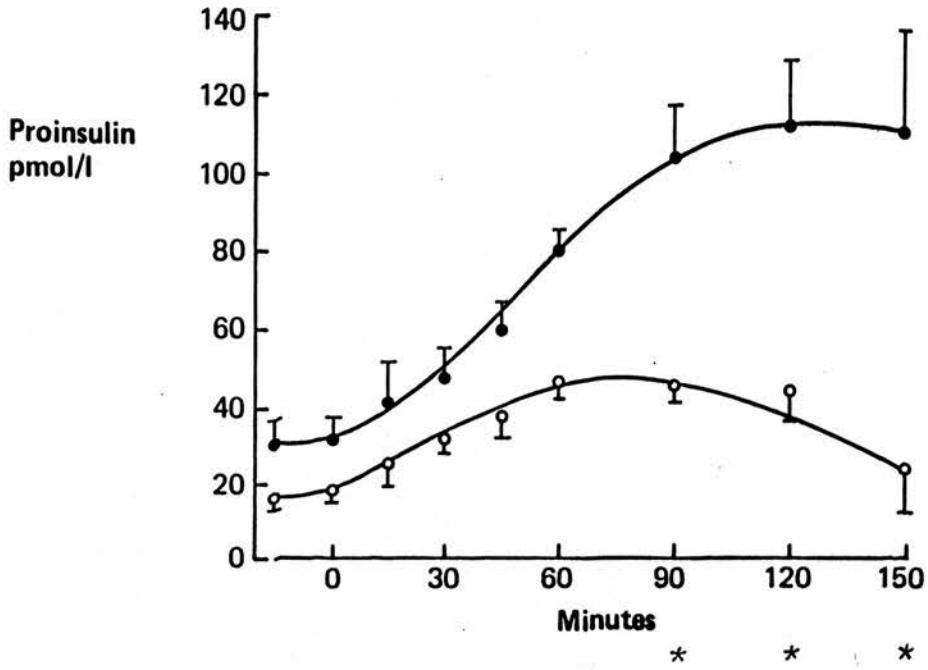

FIGURE 5.2 Mean serum proinsulin levels during glucose tolerance test.

Uraemic n = 9 (●——●) and control group n = 6 (o——o).

* $p < 0.05$

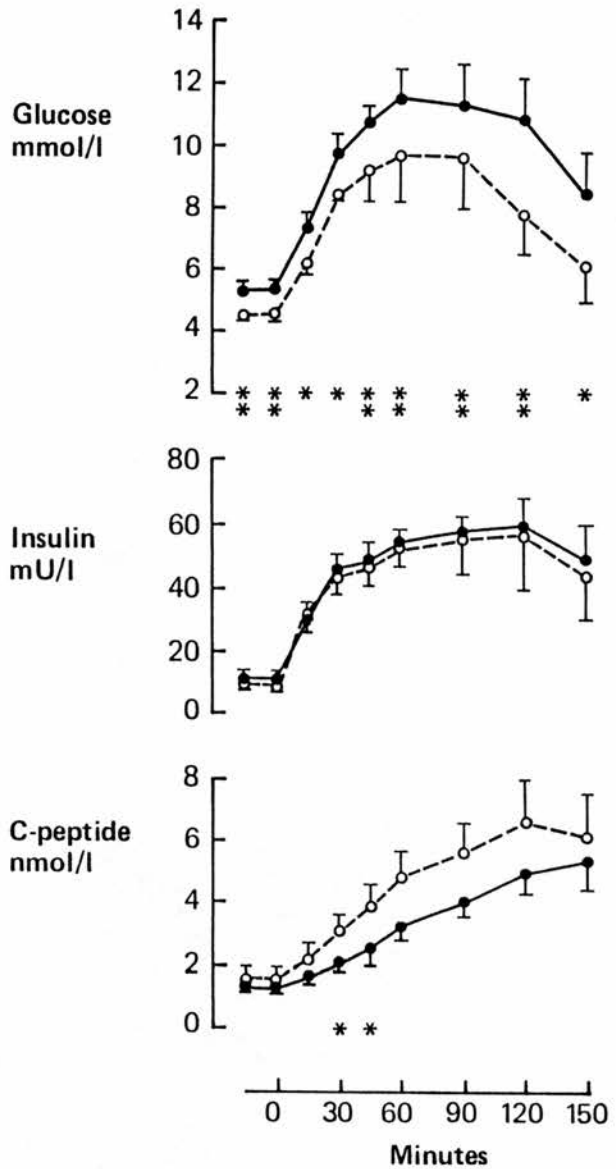

FIGURE 5.3 Mean blood glucose, serum I.R.I. and serum C-peptide responses to a 75 gram oral glucose load. Uraemic subjects (n = 5) before (●——●) and after C.A.P.D. (o——o).

* $p < 0.05$ ** $p < 0.001$

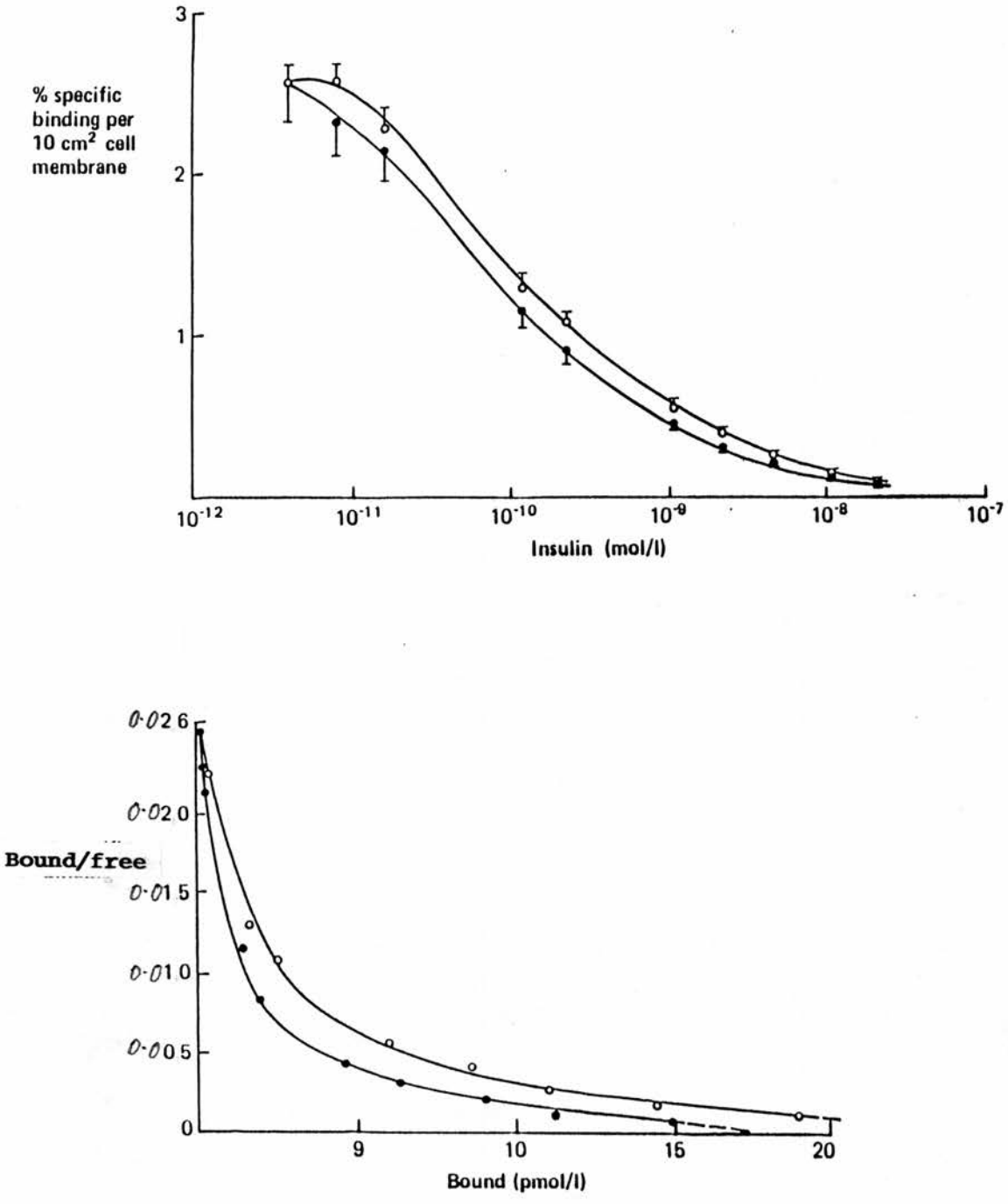

FIGURE 5.4 Specific insulin binding to adipocytes shown as binding displacement curves and Scatchard plots. Uraemic (●——●) and control (o——o) subjects.

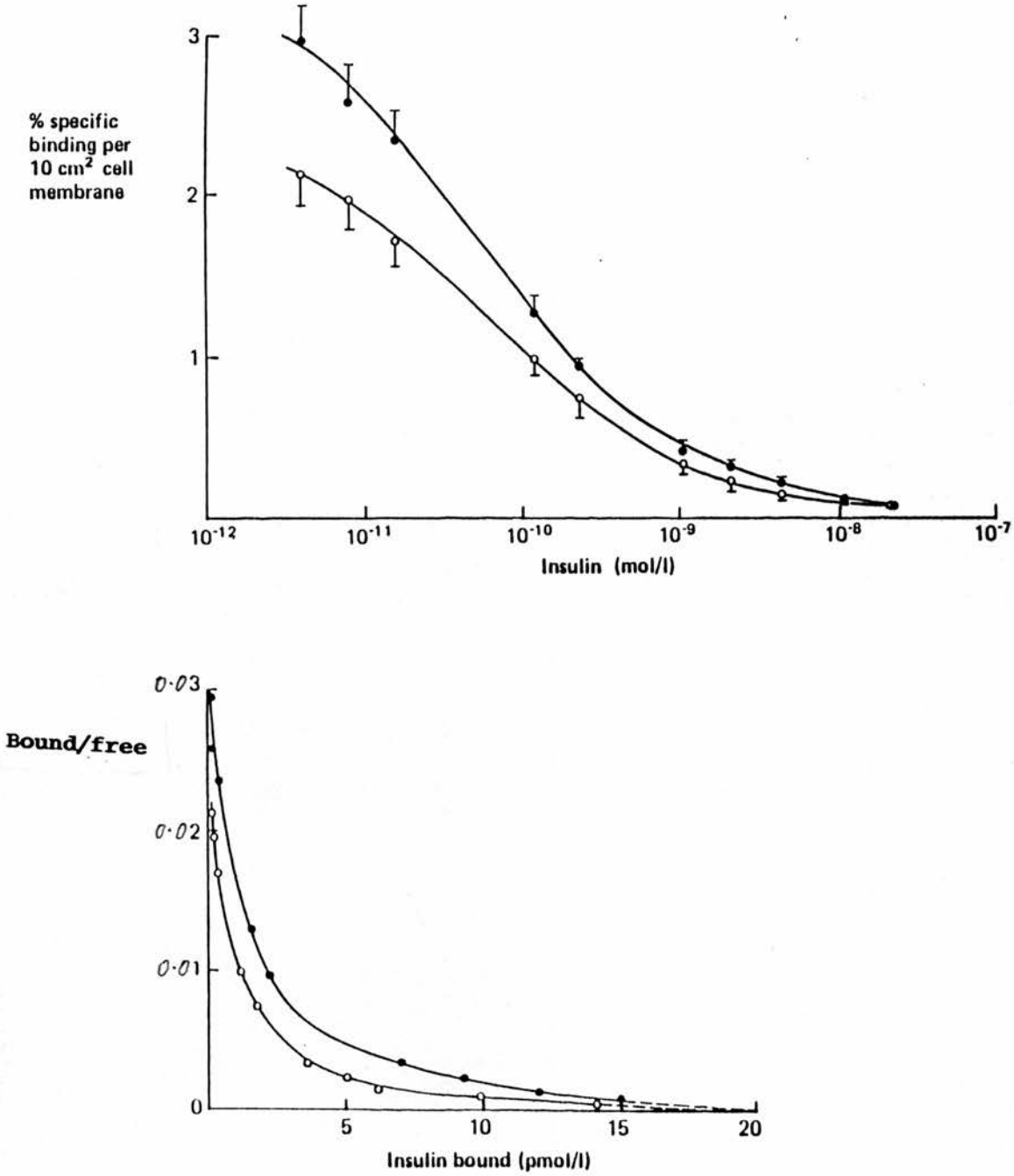

FIGURE 5.5 Specific insulin binding to adipocytes shown as binding displacement curves and Scatchard plots. Uraemic subjects (n = 5) before (●——●) and after C.A.P.D. (o——o).

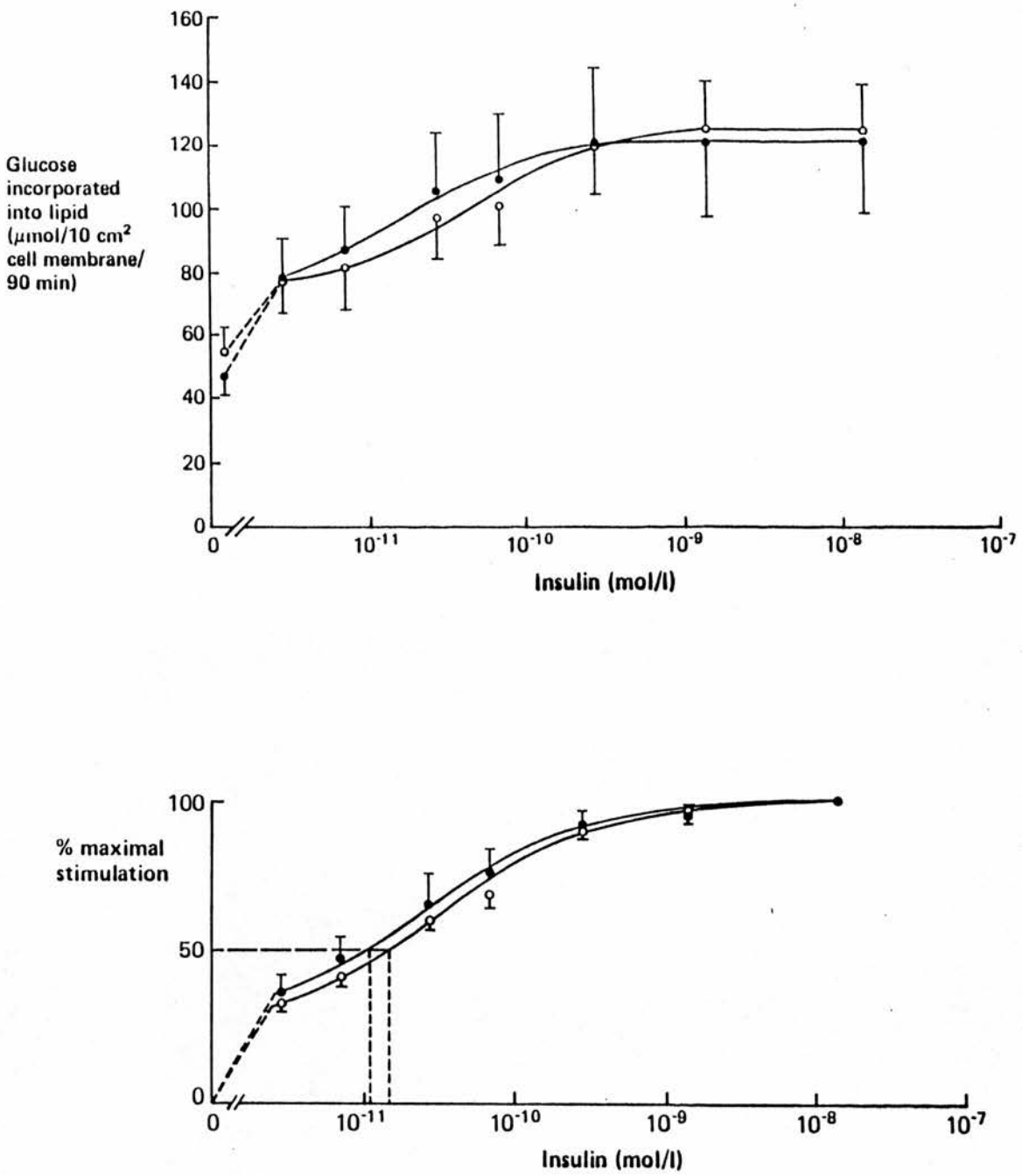

FIGURE 5.6 Insulin stimulation of lipogenesis shown as absolute rates (top) and as percentage stimulation (bottom). Uraemic n = 9 (●—●) and control n = 12 (o—o) subjects.

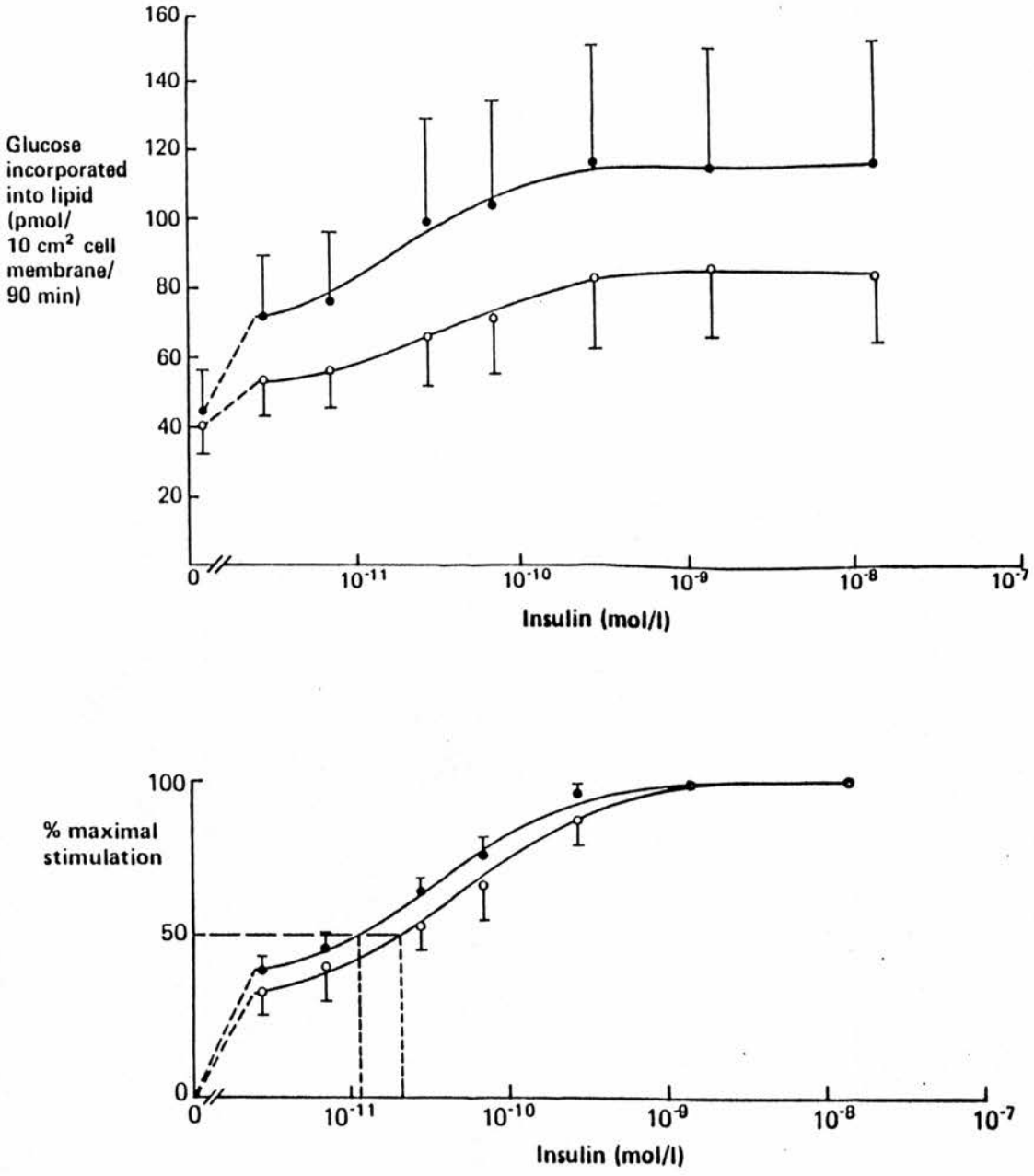

FIGURE 5.7 Insulin stimulation of lipogenesis shown as absolute rates (top) and as percentage stimulation (bottom). Uraemic subjects (n = 5) before (●——●) and after C.A.P.D. (o——o).

DISCUSSION

The uraemic group exhibited the anticipated fasting hyperglycaemia associated with normal serum insulin levels. This suggestion of insulin resistance was borne out by the prolonged hyperglycaemia despite marked hyperinsulinaemia in the last hour of the glucose tolerance test. However, peak serum I.R.I was lower and delayed in the uraemic group. The slower rate of gastric emptying and hence slower intestinal absorption which has been documented in uraemia could partially account for these changes (Craig et al 1983), but the possibility remains of blunted insulin secretory capacity. It has been reported that the first phase insulin secretion is impaired in chronic renal failure (Hampers et al 1966) but hyperglycaemic clamp studies found normal insulin secretion (DeFronzo et al 1982). Measurement of C-peptide does not resolve the question of the normality of insulin secretion rates as C-peptide clearance is grossly impaired in chronic renal failure and this results in markedly elevated levels both fasting and after the glucose load.

The fasting hyperglycaemia is most probably a consequence of lack of suppression of hepatic glucose output (DeFronzo et al 1983b) secondary to hepatic insulin resistance. Rubenfeld & Garber (1978) observed a 38% increase in fasting hepatic glucose output in uraemic subjects who exhibited the fasting hyperglycaemia observed in most studies (Neubauer 1910, Linder et al 1925, Hampers et al 1968, Spitz et al 1970, DeFronzo et al 1978a, Heaton et al 1984). However, DeFronzo has described a group of uraemic subjects of similar clinical and biochemical characteristics who had normal fasting hepatic glucose output and blood glucose levels (DeFronzo et al 1981b, Smith & DeFronzo 1982). Serum I.R.I. levels were very high in this group, and hence the possibility remains of hepatic insulin resistance in the fasting state. In the same subjects, hepatic glucose output was observed to suppress almost normally at high physiological serum insulin levels, but responsiveness rather than sensitivity was examined. In a rat model of chronic renal failure, isolated hepatocytes were shown to be insulin insensitive in respect of stimulation of amino acid uptake (Kauffman & Caro 1983).

The post-glucose hyperglycaemia observed in the present study could result from both hepatic and peripheral insulin resistance as under conditions of hyperinsulinaemia prevailing in the latter part of the glucose tolerance test, hepatic glucose output and uptake is almost normal and peripheral insulin resistance is present (DeFronzo et al 1981b, Smith & DeFronzo 1982, Westervelt 1969). In contrast to the uncertainty surrounding hepatic insulin sensitivity, peripheral insulin sensitivity has been shown to be decreased in uraemia by direct studies of human forearm or leg (Westervelt et al 1969, DeFronzo et al 1981b) and by the euglycaemic clamp technique (DeFronzo et al 1981b, Smith & DeFronzo 1982). The in vivo assessment of insulin-mediated glucose disposal is approximately 85% dependent upon muscle and 15% dependent upon adipose tissue (DeFronzo et al 1981a). Muscle and adipose tissue are however exposed to identical controlling influences and adipocytes have been observed to exhibit the insulin resistance predicted from in vivo studies (Olefsky 1976, Kolterman et al 1981, Pedersen et al 1981c, Hjollund et al 1983c). The present observations of normal maximum specific insulin binding and normal adipocyte insulin sensitivity in chronic renal failure were therefore unexpected.

Specific insulin binding to adipocytes from uraemic subjects was normal because increased affinity of the receptors compensated for the low receptor number. Several clinical states, such as hypocaloric dieting in obesity (Beck-Nielsen et al 1979a), Cushing's syndrome (Muggeo et al 1983a) and acromegaly (Muggeo et al 1979 & 1983b) have been shown to be associated with increased monocyte insulin receptor affinity. The dietary intake of the uraemic subjects was only marginally and not significantly lower than that of the normal subjects in the present study. Mean diurnal serum total cortisol level is elevated in uraemia, but this could be secondary to binding protein abnormalities (Heaton et al 1984). Serum growth hormone has been reported to be raised in some studies (Orskov & Christensen 1971) but not in others (Heaton et al 1984). Neither serum cortisol nor growth hormone levels correlate with glucose intolerance or insulin resistance (DeFronzo et al 1983, Heaton et al 1984). Hence, the observed change in receptor affinity cannot be attributed to any single recognised influence. Hyperinsulinaemia is the obvious factor

to account for the reduced receptor number. Although the decrease in receptor number cannot be ascribed to increase in fasting serum insulin levels, mean diurnal serum insulin levels are markedly raised in uraemic subjects (Heaton et al 1984). There was no correlation between fasting serum insulin and high or low affinity receptor numbers. Insulin receptor number in uraemia has been reported to be decreased on erythrocytes (Gambhir et al 1980 & 1981) but normal on monocytes (Smith & DeFronzo 1982). In the latter study, the limited range of insulin concentrations studied precluded any conclusions about number or affinity of the high affinity receptor population. In animal models of chronic renal failure, both normal affinity and receptor number on adipocytes (Maloff et al 1983) and increased receptor number on hepatocytes (Kauffman & Caro 1983) have been reported.

In the present study, increased affinity of a decreased receptor population was observed to be associated with normal in vitro adipocyte insulin sensitivity as assessed by stimulation of lipogenesis. It may be considered that the observation of normal adipocyte insulin sensitivity in a clinical state known to be associated with muscle insulin resistance in vivo constitutes the first described example of differential tissue behaviour. This could be so, but the evidence is indirect. An alternative explanation would invoke the existence of factors which only operate in vivo, confounding the interpretation of in vitro studies. Circulating inhibitors of insulin action in uraemia have been described (Dzurik et al 1969, Milutinovic et al 1983, Lockwood & McCaleb et al 1983), but the nature of such a substance remains obscure. The slight but chronic metabolic acidosis of uraemia could increase insulin resistance (Whittaker et al 1981b & 1982) and reduce receptor affinity (DeMeyts 1976). If partial compensation for the chronic effect of either a circulating inhibitor or adverse physical conditions were to develop at the cellular level, it is likely that such an effect could persist during the period of in vitro study. All cells were handled in the same supporting buffer at pH 7.42 in vitro. Sudden removal of the inhibitory influence could lead to the cells being more sensitive under the experimental conditions than under habitual in vivo conditions.

After three months C.A.P.D. glucose tolerance was improved although not returned to normal. The glucose tolerance curve was shifted downwards but unchanged in shape, peak blood glucose levels still being observed at 60 minutes compared to 30 minutes in the normal control group. Serum I.R.I. levels both fasting and after the glucose load were almost identical before and after C.A.P.D., suggesting that the improvement in glucose tolerance was secondary to improved tissue senstivity to insulin, not improved pancreatic B cell function. The fall in fasting blood glucose levels despite almost identical serum insulin levels suggest that an improvement in hepatic insulin sensitivity had occured during C.A.P.D. The slightly higher fasting and post-glucose C-peptide levels could possibly be an effect of further decline in renal function during the months of dialysis.

The effect of C.A.P.D. upon adipocyte insulin binding was the opposite of that expected by consideration of the apparent improvement in insulin sensitivity suggested by the glucose tolerance test data. C.A.P.D. brings about changes in nutrition, acid-base balance, serum lipids, physical activity and general well-being (Ramos *et al* 1983). One possible explanation of the decrease in adipocyte insulin binding could be that patients on C.A.P.D. are never in the fasting state, peritoneal glucose absorption continuing day and night. This induces persisting hyperinsulinaemia (Heaton *et al* 1984) which could down-regulate insulin receptors.

Despite the decrease in adipocyte insulin binding, *in vitro* insulin sensitivity did not change significantly in the five patients restudied. Changes in the post-binding pathways of insulin action, counteracting the effect of decreased receptor binding may be inferred. The glucose tolerance test data suggest improvement in insulin sensitivity during C.A.P.D. which is likely to involve both hepatic and peripheral tissues. If a circulating inhibitor of insulin action had been at least partly removed by dialysis, then the insulin sensitivity observed *in vitro* could well reflect more closely that operating in vivo, unlike the situation before dialysis.

In conclusion, normal insulin sensitivity and responsiveness have been observed in adipocytes from subjects with advanced chronic

renal failure. This contrasts sharply with *in vivo* muscle insulin resistance, and could be a result of the operation of circulating inhibitors of insulin action. The possibility exists of differential tissue behaviour with respect to insulin action *in vivo*. Adipocyte insulin receptors have been shown to be of higher than normal affinity but reduced in number. C.A.P.D. brought about a decrease in receptor affinity and in responsiveness to insulin, and adipocyte insulin sensitivity remained normal. Glucose tolerance improved during C.A.P.D. secondary to improved hepatic, and possibly peripheral, insulin sensitivity. These data describe an unusual adipocyte insulin receptor status and reflect the complexity of the response of different tissues to the state of chronic renal failure.

CHAPTER SIX

HYPERTHYROIDISM

INTRODUCTION

Hyperthyroidism is associated with marked metabolic disturbances. Fasting hyperglycaemia has been reported in some studies of hyperthyroid man (Ikejiri et al 1978, McCulloch et al 1982, McCulloch 1983a) but others have not confirmed this (Hales & Hyams 1964, Saunders et al 1980). Hyperglycaemia following glucose or mixed meals has frequently been described (Hales and Hyams 1964, Ikejiri et al 1978, Saunders et al 1980, McCulloch et al 1982). Fasting plasma non-esterified fatty acid and glycerol concentrations are elevated in hyperthyroidism (McCulloch et al 1983a). The processes underlying these alterations in circulating concentrations of glucose, non-esterified fatty acids and glycerol have been extensively investigated.

Increased hepatic glucose output could cause fasting hyperglycaemia and has been demonstrated (Saunders et al 1980, Perez et al 1980, McCulloch et al 1983b) in all but one study (Wahren et al 1981). Enhanced gluconeogensis may be inferred from the observation of increased hepatic uptake of the major gluconeogenic precursors, especially glycerol (McCulloch et al 1983b). Hepatic glycogen stores are decreased in hyperthyroid man (Hoch 1962, Movitt et al 1963) and the demonstration of more rapid depletion of glycogen stores in the perfused livers of hyperthyroid rats suggests that increased glycogenolysis rather than decreased glycogen synthesis may be the mechanism (Laker & Mayes 1981). Hence, increased rates of both gluconeogenesis and glycogenolysis could contribute to an increased hepatic glucose output. Post-prandial hyperglycaemia has been suggested to be a consequence of rapid gastric empying (Holdsworth and Besser 1968), but there is no evidence to support this concept and peak blood glucose levels occur at a normal time after oral glucose or mixed meals (McCulloch et al 1982). Reduced hepatic extraction of glucose is a possible factor, secondary either to reduced fractional extraction and normal hepatic blood flow, or to increased hepatic blood flow with normal or reduced fractional extraction (Wahren et al 1981). Increased peripheral tissue lipolysis is associated with increased turnover rates of both non-esterified fatty acids and glycerol (Saunders et al 1980, McCulloch et al

1983b).

The altered regulatory mechanisms which bring about the characteristic abnormalities of hyperthyroidism are at present uncertain. The enhanced adipose tissue lipolysis has been suggested to be a result of increased sensitivity to catecholamines (Christensen 1973), but beta-blockade does not suppress this (Beylot et al 1980, Saunders et al 1980). Some hyperthyroid subjects have elevated plasma glucagon levels with impaired suppression following an oral glucose load (Kabadi & Eisenstein 1980). Reduced insulin action could theoretically precipitate all of the observed abnormalities. Measurement of serum I.R.I. has dismissed the concept of a hypoinsulinaemic state, fasting serum I.R.I. concentration having been found to be normal (Holdsworth & Besser 1968, McCulloch et al 1982, McCulloch et al 1983) or elevated (Doar et al 1969), with the I.R.I. response to an oral glucose load being normal. The observation of normal or high levels of circulating I.R.I. in the face of impaired glucose tolerance and unrestrained lipolysis has raised the possibility of hepatic and peripheral insulin resistance. Examination of insulin sensitivity in hyperthyroid man has produced conflicting results, both enhanced (Elrick et al 1961) and decreased (West et al 1975, Ikerjiri et al 1977) insulin sensitivity having been reported.

Direct examination of segments of adipose tissue from hyperthyroid subjects suggested decreased sensitivity to insulin, the dose-response curve for anti-lipolysis being right-shifted, but with a normal maximal response (Wennlund et al 1981). However, isolated adipocytes from hyperthyroid rats were found to have not only normal sensitivity and responsiveness to insulin stimulation of lipogenesis, but also greatly increased numbers of insulin receptors (Heise et al 1982).

In view of the possibility of peripheral tissue insulin resistance contributing to the metabolic abnormalities of hyperthyroidism, the present studies were designed to elucidate the insulin sensitivity and insulin receptor status of adipocytes from patients with hyperthyroidism, and to relate this to the pattern of

insulin secretion in response to oral glucose.

SUBJECTS AND PROTOCOL

Thirteen patients referred to the clinic with a provisional diagnosis of hyperthyroidism were recruited to the study. No prior treatment had been given to any subject. The subjects were subsequently divided into hyperthyroid and control groups on the basis of total serum thyroxine and thyrotrophin releasing hormone tests. The control subjects had no evidence of any organic disease which may have produced the presenting symptoms, and thus constituted a group of subjects who had similar symptoms of adrenergic overactivity but who did not have elevated serum thyroid hormone levels. The clinical and biochemical characteristics of the two groups are listed in Table 6.1. Fasting adipocyte tissue biopsies and oral glucose tolerance tests were performed on each subject within a three day period.

RESULTS

a) Oral glucose tolerance tests

Mean fasting blood glucose was 4.9 $\pm$ 0.3 mmol/l for the hyperthyroid groups compared with 4.4 $\pm$ 0.2 mmol/l for the control group ($p < 0.1 > 0.05$). The rise in blood glucose was greater in the hyperthyroid group, being significantly different at 10, 25, 30, 45 and 90 minutes ($p < 0.05$) (Figure 6.1). Fasting serum I.R.I. levels were similar in the two groups (9.5 $\pm$ 2.8 vs 7.5 $\pm$ 0.8 mU/l for hyperthyroid and control groups respectively). Peak serum I.R.I. was 69.0 $\pm$ 6.8 mU/l for the hyperthyroid group (at 45 min) and 54.3 $\pm$ 8.8 mU/l for the control group (at 60 min)($p < 0.1 > 0.05$). Fasting serum proinsulin was very similar in the two groups (14.1 $\pm$ 3.8 vs 15.8 $\pm$ 3.2 pmol/l respectively) but rose more steeply to a higher peak in the hyperthyroid group (64.8 $\pm$ 7.3 vs 39 $\pm$ 3.7 pmol/l at 60 min; $p < 0.01$) (Figure 6.2). Correction of measured I.R.I. for proinsulin cross-reactivity in the insulin assay further diminishes the

difference in mean peak I.R.I. levels. Serum C-peptide levels were similar both basally (0.46 ± 0.11 vs 0.37 ± 0.04 nmol/l respectively) and throughout the oral glucose tolerance test (Figure 6.1).

b) Adipocyte insulin binding

The binding displacement curve is shown in Figure 6.3. Maximum specific insulin binding was 1.80 ± 0.18% for the hyperthyroid group and 2.62 ± 0.27% for the control group ($p < 0.025$). The difference in specific insulin binding was significant at all points up to an insulin concentration of 2.2 nmol/l insulin. Half-maximum binding was observed at total insulin concentrations of 145 ± 16 and 120 ± 8 pmol/l respectively ($p > 0.1$). Scatchard analysis of this data yielded parallel plots (Figure 6.3) suggesting that the difference in binding was secondary to reduced receptor number rather than any alteration of affinity. There was a negative correlation between maximum specific binding and total serum thyroxine within the hyperthyroid group ($R_s = 0.71$, $p < 0.05$) (Figure 6.4).

c) Adipocyte insulin sensitivity

The rates of adipocyte lipogenesis were 52.0 ± 16.0 and 87.0 ± 14.4 pmol/10 cm^2/90 min basally for the hyperthyroid and control groups respectively ($p < 0.1 > 0.05$) and 121.5 ± 28.0 and 175.8 ± 25.4 pmol/10cm^2/ 90 min with maximal insulin stimulation respectively ($p > 0.1$). Insulin stimulation achieved similar percentage increases above basal levels in each group and the insulin concentrations required to achieve half-maximal stimulation were similar (13.9 ± 3.6 vs 11.4 ± 2.1 pmol/10 cm^2/90 min respectively) (Figure 6.5).

The basal rate of glucose transport was 0.75 ± 0.22 pmol/10 cm^2/20 sec for the hyperthyroid subjects studied (n = 3) and 1.00 ± 0.22 pmol/10cm^2/ 20 sec for the control subjects studied (n = 4). The insulin concentrations required to achieve half-maximal stimulation were almost identical (24.3 ± 2.2 vs 24.6 ± 3.6 pmol/10 cm^2/20 sec respectively) (Figure 6.6).

	THYROTOXIC n = 7	CONTROL n = 6
Sex	Female	Female
Age (years)	53.4 ± 3.5	41.5 ± 4.8
% I.B.W.	100 ± 4	108 ± 9
Adipocyte diameter (μ)	97 ± 5	105 ± 7
Diet - grams carbohydrate /day	208 ± 14	200 ± 18
Diet - kcal /day	1904 ± 160	1958 ± 116
Total serum thyroxine (nmol/l)	252 ± 25	117 ± 9

TABLE 6.1 Clinical and metabolic characteristics of the hyperthyroid and control groups. The only significant difference was that in serum thyroxine levels ($p < 0.001$).

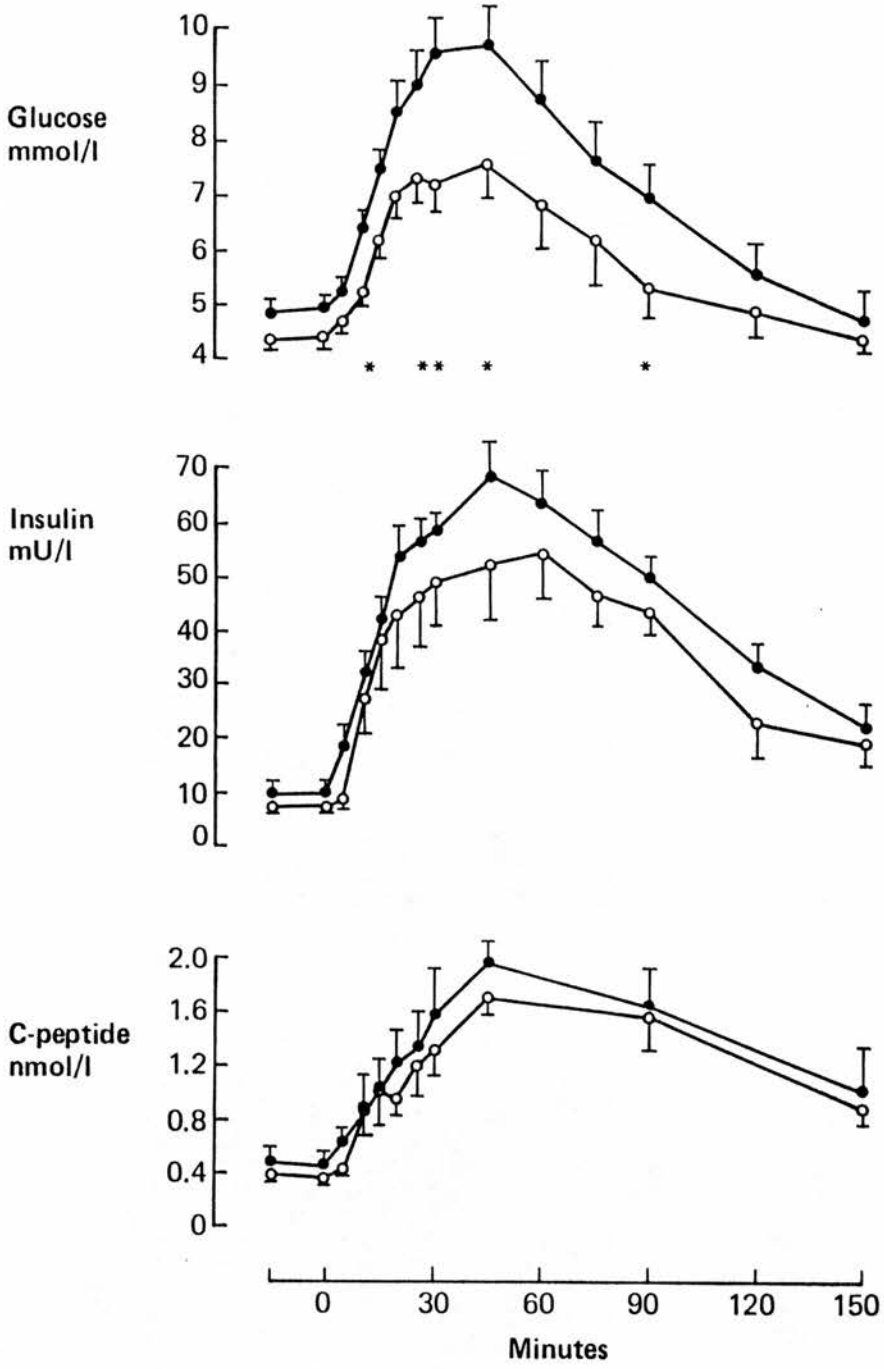

FIGURE 6.1 Mean blood glucose, serum I.R.I. and serum C-peptide responses to a 75 gram oral glucose load.
Hyperthyroid (●——●) and control (o——o) groups.
* $p < 0.05$

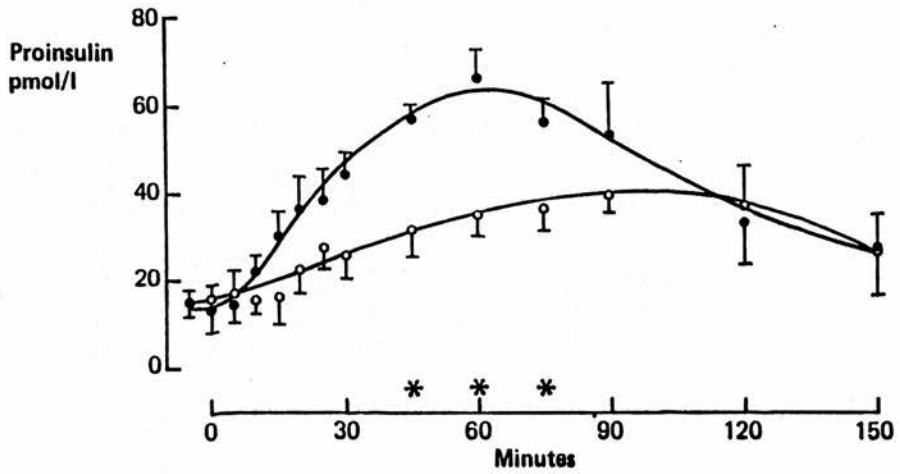

FIGURE 6.2 Mean serum proinsulin levels during the glucose tolerance test.

Hyperthyroid (●—●) and control (o—o) subjects.

* $p < 0.05$

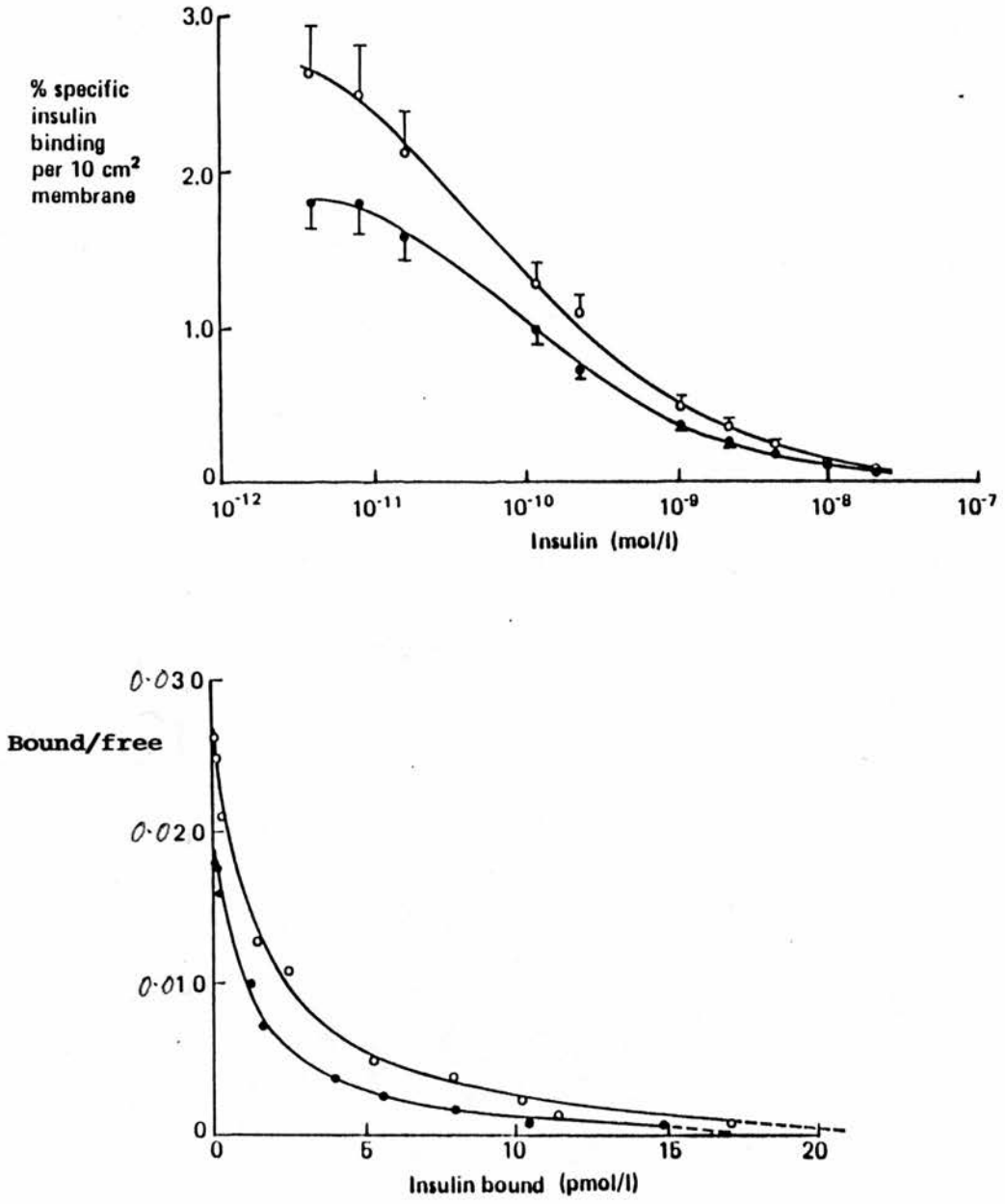

FIGURE 6.3 Specific insulin binding to adipocytes shown as binding displacement curves and Scatchard plots. Hyperthyroid (●——●) and control (o——o) subjects.

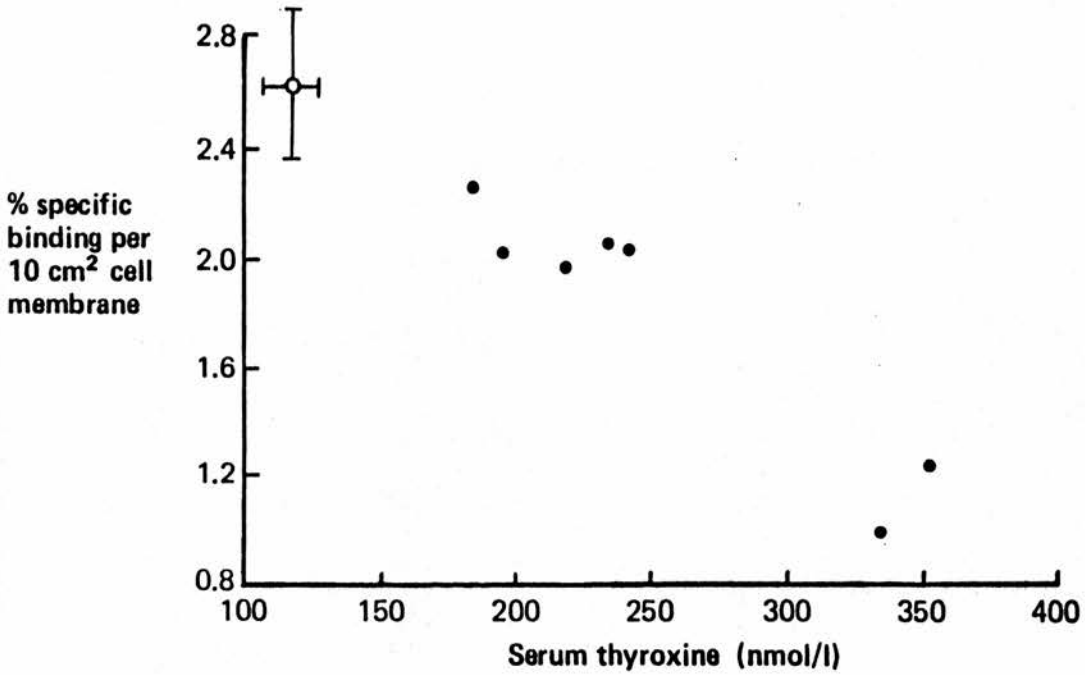

FIGURE 6.4 Relationship between specific insulin binding to adipocytes and total serum thyroxine in the hyperthyroid group (•). $R_s = 0.71$; $p < 0.05$. Control data is shown as mean ± SEM (o).

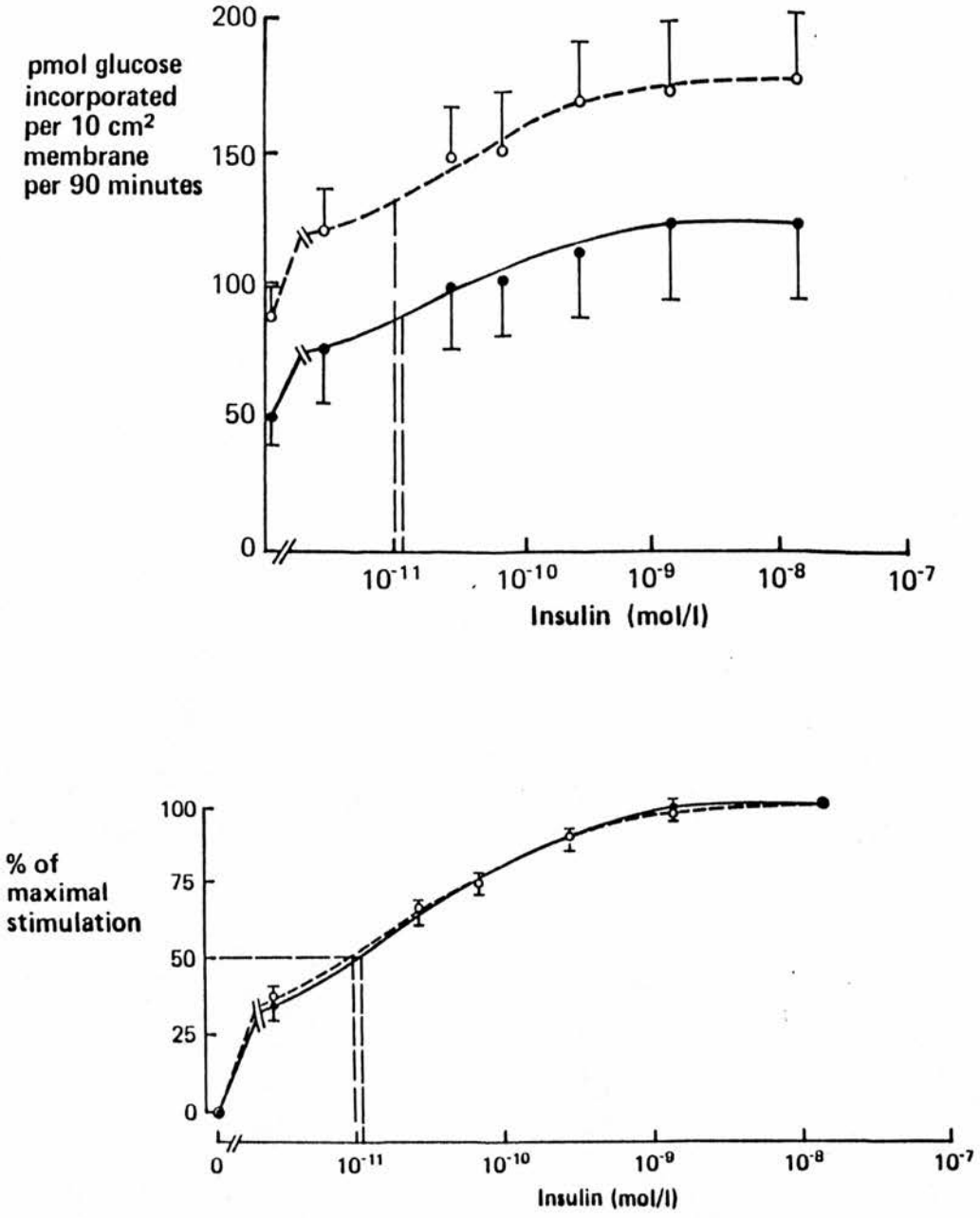

FIGURE 6.5 Insulin stimulation of lipogenesis in adipocytes shown as absolute rates (top) and as percentage stimulation (bottom). The dotted line indicates the insulin concentrations at which half-maximal stimulation were observed.

Hyperthyroid (●——●) and control subjects (o——o).

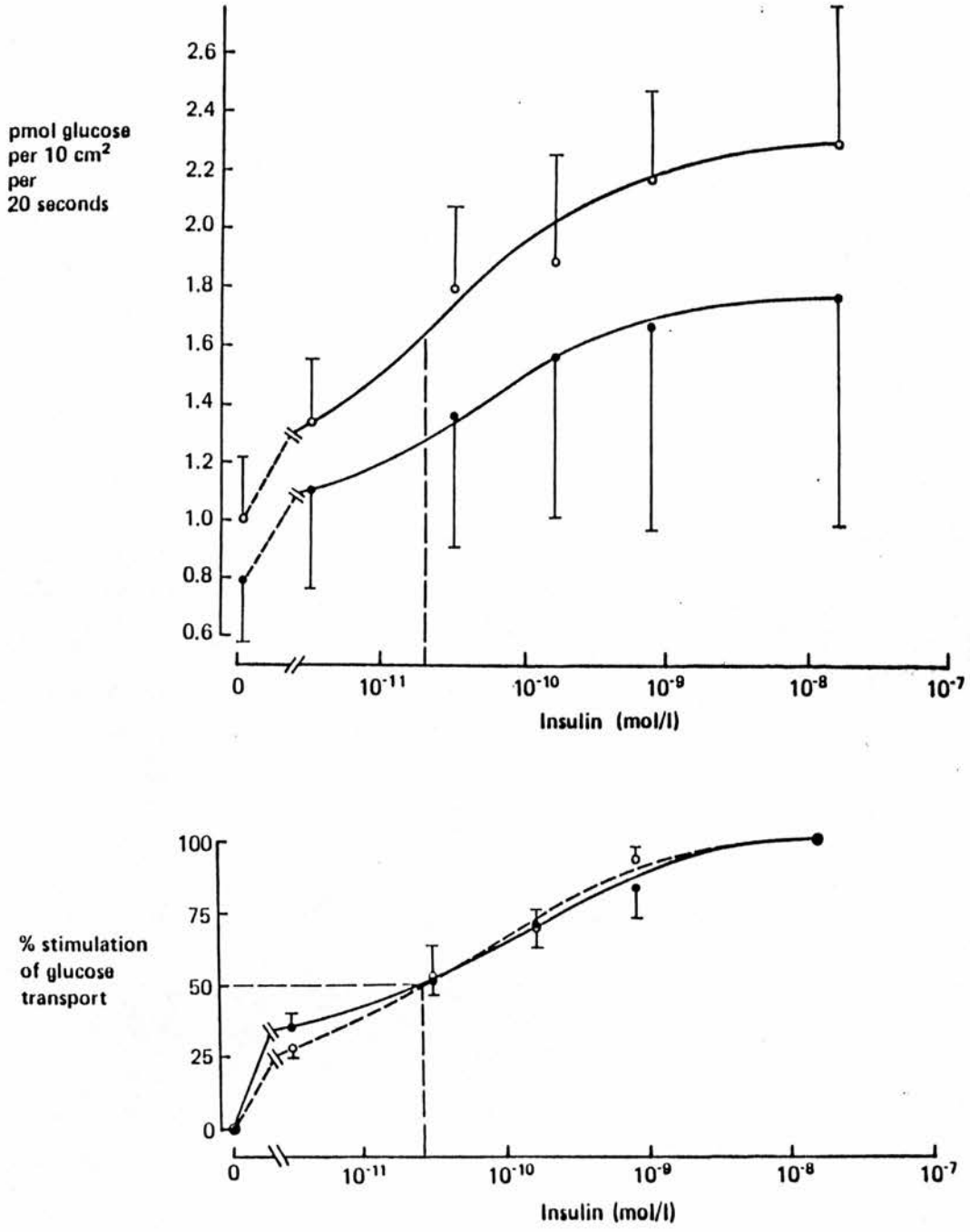

FIGURE 6.6 Insulin stimulation of glucose transport in adipocytes shown as absolute rates (top) and as percentage stimulation (bottom). The dotted line indicates the insulin concentrations at which half-maximal stimulation were observed. Hyperthyroid (●——●) and control subjects (o——o).

DISCUSSION

In previous studies of hyperthyroid patients, elevated fasting and post-prandial blood glucose concentrations (McCulloch et al 1982a) and increased glucose production rates (McCulloch et al 1983b) have been observed despite normal serum insulin levels. The present study was designed to investigate insulin secretion, tissue insulin receptor status and insulin sensitivity in hyperthyroid and normal subjects.

Hyperglycaemia following oral glucose administration was observed in this as in many previous studies. However, serum insulin levels were not significantly different in the two groups. It has been suggested that much of the insulin measured by standard radioimmunoassay in hyperthyroidism may in fact be proinsulin (Sestoft & Heding 1981). Proinsulin hypersecretion in hyperthyroidism was observed in the present study, and as proinsulin was found to exhibit 50% crossreactivity with insulin in the insulin assay used, it is apparent that the observed peak immunoreactive insulin levels would be affected by the proinsulin present. The true insulin levels are closer in the two groups than the I.R.I. levels suggest. The finding of normal C-peptide responses to oral glucose in the present study confirms that the insulin secretion itself was normal, insulin and C-peptide being secreted in equimolar amounts. Hence, the hyperthyroid subjects displayed resistance to insulin action either in promoting hepatic glucose uptake, in inhibiting hepatic gluconeogenesis or in promoting glucose uptake into the peripheral tissues, muscle and fat.

The adipocyte insulin binding studies demonstrated that insulin receptor number was decreased in hyperthyroidism, corroborating recently reported observations (Arner et al 1984a). This finding is in sharp contrast to the demonstration that the adipocytes of rats rendered acutely hyperthyroid almost doubled their insulin receptor number (Heise et al 1982). The possiblity of a species difference cannot be dismissed, but studies after more prolonged periods of thyroxine administration to rats would be of interest. The time course of the cellular insulin receptor response to continued

stimulation by thyroid hormones requires further study in view of the possibility of initial increase in synthesis rate or recruitment from a latent pool of insulin receptors (Deutsch et al 1982) followed by compensatory downregulation. To date, few factors have been shown to be involved in the physiological control of insulin receptor number, insulin itself being by far the most powerful influence (Gavin et al 1974, Marshall & Olefsky 1980, Pezzino et al 1980). The results of the present study suggest that thyroid hormones may be involved in the control of insulin receptor number. The inverse correlation between total serum thyroxine and adipocyte insulin binding, which was also observed by Arner and colleagues (1984a), adds weight to this hypothesis.

Adipocyte insulin resistance is usually present in those clinical states associated with reduced adipocyte insulin receptor number, such as obesity (Olefsky 1976, Pedersen et al 1981c), diabetes (Pedersen & Hjollund 1982, Olefsky et al 1982a) and cirrhosis (Taylor et al 1984a). However, the insulin concentrations required to achieve half-maximal stimulation of both glucose transport and lipogenesis were similar for the adipocytes of the hyperthyroid and control groups. The adipocytes from the hyperthyroid subjects thus exhibited not only receptor downregulation but also compensation for the receptor alteration at the post-binding level. A dissociation of biological effects from change in adipocyte insulin receptor number was also observed after acute thyroxine administration to rats (Heise et al 1982), although in this case insulin receptor number increased without change in adipocyte insulin sensitivity. The explanation for this phenomenon is obscure but fuels the concept that the linkage between receptor binding and insulin action is not simple. The observation that adipose tissue from hyperthyroid subjects did not exhibit normal sensitivity to insulin inhibition of lipolysis and stimulation of total glucose oxidation (Wennlund et al 1981) suggests that the regulation of these post-binding pathways in hyperthyroidism differs from that of the glucose transport and lipogenesis pathways. The possiblity of peripheral resistance to insulin action has recently been investigated in vivo using the glucose clamp technique (McCulloch et al 1983a). The stimulation of glucose disposal by exogenously administered porcine

insulin was identical in hyperthyroid and control subjects but there was resistance to suppression of lipolysis during insulin infusion (McCulloch *et al* 1983a) implying differential regulation of the post-binding pathways of insulin action.

An alternative explanation of the insulin action data could take into account the absolute basal rates of lipogenesis and glucose transport. The basal rates of both processes appear to be lower in the hyperthyroid group, albeit not statistically significantly with the small numbers studied. It is possible that the measured basal rates *in vitro* reflect a reduced tonic stimulation *in vivo* as a result of normal tissue insulin concentrations acting through reduced numbers of insulin receptors. The enzymes responsible for lipogenesis may be expected to be less induced than in normal cells. If any given insulin-generated signal reaching the cell cytoplasm brings about a certain absolute increase in *in vitro* rates of lipogenesis or glucose transport then the effect on the adipocytes of the hyperthyroid group would be smaller, being mediated through reduced receptor numbers. The same insulin concentration would be expected to bring about a greater increase in non-hyperthyroid adipocytes. However, the percentage increase may be similar in both cases because of the difference in basal rates. In this manner, the use of the traditional measure of insulin sensitivity may mask the functional effects of the low insulin receptor number in the *in vitro* system. There is, however, no direct evidence to confirm this hypothesis, and the former interpretation of the data must be favoured.

The apparently normal sensitivity of adipocytes from hyperthyroid subjects to insulin stimulation of glucose transport and lipogenesis accords with the recent demonstration of normal peripheral tissue sensitivity to exogenously administered insulin in man (McCulloch *et al* 1983a). The elimination of peripheral tissue resistance to glucose disposal as a possible cause of post-prandial hyperglycaemia makes defects of hepatic insulin sensitivity in hyperthyroidism more likely. Indirect support for this hypothesis is provided by the observation that hepatic glycogen stores are markedly reduced in hyperthroidism (Hoch 1962, Maracek & Feldman 1973) and do not increase after oral glucose (Mirsky & Broh-Kahn 1936). The only

study of insulin binding to liver membranes from hyperthyroid rats showed a 22% reduction in maximum binding, although small numbers prevented statistical significance from being achieved (DeRuyter *et al* 1982).

In conclusion, normal insulin secretory responses but relative hyperglycaemia following oral glucose have been observed in hyperthyroidism. No abnormality of adipocyte insulin sensitivity could be detected *in vitro* but adipocyte insulin receptor number was reduced. These results suggest that thyroid hormones could play a role in the control of insulin receptor number, this being modulated in such a manner that cellular sensitivity to insulin stimulation of lipogenesis and glucose transport remains unchanged. The normal insulin sensitivity of adipocytes, together with the recent *in vivo* observation of normal peripheral tissue insulin sensitivity suggest, albeit indirectly, that disturbed hepatic insulin responsiveness plays a major role in the glucose intolerance of hyperthyroidism.

CHAPTER SEVEN

BRITTLE AND STABLE INSULIN DEPENDENT DIABETES

INTRODUCTION

Peripheral hyperinsulinaemia may be regarded as a characteristic feature of insulin-dependent diabetes (Asplin *et al* 1979). Conventional subcutaneous administration of insulin in doses sufficient to achieve adequate portal vein insulin concentrations, and thus control of hepatic metabolism, must inevitably result in peripheral hyperinsulinaemia. This is likely to induce compensatory changes in insulin sensitivity and responsiveness in peripheral target organ tissues such as muscle and adipose tissue. *In vivo* studies have demonstrated decreased peripheral tissue insulin sensitivity in insulin-dependent diabetes (Harano *et al* 1981, DeFronzo *et al* 1982, Proietto *et al* 1983). However, well controlled insulin-dependent diabetic subjects, in whom the degree of hyperinsulinaemia may be expected to be more exaggerated, have less marked insulin resistance *in vivo* (Beck-Nielsen *et al* 1984, Reevers *et al* 1984). This suggests that hyperinsulinaemia is not the decisive factor *per se*, but that some aspect of metabolic control is important in determining insulin sensitivity. Although the majority of insulin-dependent diabetic subjects respond to insulin in a predictable fashion, a small number exhibit fluctuating and pronounced resistance to subcutaneously administered insulin. After exclusion of subjects with recognised causes of erratic diabetic control, there remain a small number of subjects whose metabolic contol may be described as brittle.

The term "brittle diabetes" has been applied in many different ways to states of unstable diabetic control. It was originally used to describe a group of insulin-dependent diabetic patients who experienced frequent and inexplicable oscillations between hyperglycaemic ketosis and hypoglycaemia (Woodyatt 1937). The definition was subsequently widened to include any diabetic whose life was disrupted by episodes of either hyperglycaemia or hypoglycaemia (Tattersall 1977). For the purpose of this study, the term "brittle diabetes" was restricted to those subjects who were referred for management of extremely unstable diabetes, no cause for which could be identified, and who proved impossible to control on subcutaneous insulin despite intensive in-patient supervision. This

definition, which has been used by others (Pickup et al 1983), serves to delineate those patients who present the most difficult problems of management. These patients all require high doses of insulin intramuscularly or intravenously, routes which achieve adequate plasma free insulin levels in such patients (Pickup et al 1981, Home et al 1982). Resistance to the action of insulin at the tissue level therefore could explain the poor response of brittle diabetic patients to appropriate insulin therapy.

The present studies were performed in order to test the hypothesis that the abnormal response to insulin in brittle diabetes is a consequence of pathology at the level of the insulin receptor or subsequent steps of insulin action. Adipose tissue was chosen for these studies, being metabolically important, capable of responding to insulin in vitro and readily accessible. The results of studies upon a group of brittle diabetic patients were compared with those of matched groups of stable insulin-dependent diabetic and non-diabetic subjects.

SUBJECTS AND PROTOCOL

Six insulin-dependent diabetic patients who had been referred to Newcastle upon Tyne from other centres throughout Great Britain with extremely unstable diabetes were studied. Prior to referral the patients had suffered a mean of 19 (range 5-35) episodes of severe ketoacidosis and over the six month period preceding referral had spent 80 (range 42-120) days in hospital. No cause for the unpredictable tendency to ketoacidosis could be identified, and no patient could be controlled on subcutaneous insulin despite intensive supervision. The control group of seven stable insulin-dependent diabetic subjects were chosen to match the brittle group with respect to age, sex, body weight and duration of diabetes (Table 7.1). Only subjects with sub-optimal diabetic control were selected. A group of young non-diabetic female subjects were also studied. The brittle group were receiving more than twice the daily insulin dose of the stable group, three by intramuscular injection and three by intravenous infusion. All the control group were receiving

subcutaneous insulin, one by continuous infusion and six by intermittent injection of short and intermediate acting insulins. Both diabetic groups were receiving carbohydrate restricted diets. The brittle diabetic patients and one stable diabetic patient were studied as in-patients, and six of the stable diabetic subjects were studied as outpatients. Fasting fat biopsies were performed on each subject after at least three days free of marked fluctuations in diabetic control. Three brittle diabetic patients were restudied after a period of three to nine months of stable diabetic control. This was achieved in one subject by use of an external insulin infusor (Siemens) delivering into the peritoneal cavity and in two patients by use of a totally implanted pump (Infusaid) delivering intravenously in one patient and intraperitoneally in the other.

RESULTS

a) Insulin Binding

The binding displacement curves are shown in Figure 7.1. Insulin binding at tracer insulin concentrations was significantly reduced in the brittle group (1.78 $\pm$ 0.18 % per 10 cm^2 cell membrane) compared with the stable diabetic group (2.57 $\pm$ 0.36 % per 10 cm^2 cell membrane; $p < 0.05$) who themselves were not significantly different from control subjects (Table 7.2). The difference in binding between brittle and stable diabetic patients was significant at all insulin concentrations up to 120 pmol/l. Half-maximum displacement of tracer insulin was observed at 206 $\pm$ 42 for the brittle group and 122 $\pm$ 13 pmol/l for the stable group ($p = 0.069$). This suggestion of reduced receptor affinity in the brittle group was borne out by Scatchard analysis (Figure 7.1). The slope of the high affinity portion of the Scatchard plot for the brittle group was found to be reduced but the receptor number, as assessed from the x axis intercepts, was similar in the two groups. Adipocyte insulin binding data for the normal control group is summarized in Table 7.2.

b) Adipocyte insulin degradation

Total insulin degradation was similar in the presence of cells of the brittle, stable diabetic and normal groups (1.14 $\pm$ 0.04%, 1.36 $\pm$

0.12% and 1.21 ± 0.11% respectively at 60 minutes and 2.41 ± 0.17%, 2.66 ± 0.21% and 2.42 ± 0.10% respectively at 120 minutes; Figure 7.2). Tracer insulin degradation in the presence of cold insulin (10 μmol/l) did not increase markedly between 60 and 120 minutes whereas the increase in tracer insulin degradation in the absence of cold insulin increased linearly with time.

c) Adipocyte insulin sensitivity

Basal and maximal rates of lipogenesis were similar in the two diabetic groups (75 ± 14, 181 ± 30 and 61 ± 5, 187 ± 37 pmol/l per 10 cm^2 per 90 min for brittle and stable groups respectively) (Figure 7.3). The insulin concentration required to achieve half-maximal stimulation was 34 ± 4 pmol/l for the brittle group and 14 ± 3 pmol/l for the stable group ($p < 0.01$). The lipogenesis data for the stable diabetic group were similar to those of the normal control group in all respects (Table 7.2).

d) Relationship between adipocyte insulin binding and insulin sensitivity

There was a negative correlation between maximum specific binding to adipocytes and the insulin concentration required to achieve half-maximal stimulation of lipogenesis (Figure 7.4) both for all subjects ($R_s = 0.78$, $p < 0.01$) and for the brittle diabetic subjects alone ($R_s = 0.89$, $p < 0.05$).

e) Effects of achieving stable control

In the first patient who was studied twice, maximum specific insulin binding increased secondary to a change in receptor affinity and this was accompanied by an increase in insulin sensitivity in vitro, the insulin concentration required to achieve half-maximal stimulation of lipogenesis after intraperitoneal insulin being almost identical to that for the stable diabetic group (Figure 7.5 and Table 7.3). In the other two patients, adipocyte receptor affinity appeared to rise but maximum specific insulin binding did not change markedly (Table 7.3). Adipocyte insulin sensitivity, as defined by the insulin concentrations necessary to achieve half-maximal stimulation of lipogenesis, increased in all three subjects although maximally stimulated rates of lipogenesis appeared to decrease.

	BRITTLE DIABETIC PATIENTS (n=6)	STABLE DIABETIC PATIENTS (n=7)	NORMAL SUBJECTS (n=6)
Sex		ALL FEMALE	
Age (years)	22±2	25±2	35±4
Ideal body weight (%)	128±8	132±5	112±9
Mean adipocyte diameter (μ)	107±6	110±3	106±7
Duration of diabetes (years)	10.6±3.0	14.3±2.0	-
Insulin dose (units/day)	134±17	57±6	-
Fasting plasma free insulin (mU/l)	41.3±18.2	11.2±1.4	8.0±0.7
HbA_1 (%)	12.6±1.3	11.1±0.5	-
Diet - grams carbohydrate/day	112±6	131±14	178±24
- Kcal/day	1146±81	1477±166	2010±126
- grams fat/day	67±5	79±7	98±1

TABLE 7.1 Clinical characteristics of the brittle diabetic, stable diabetic and normal groups. There was no significant difference between the brittle and stable diabetic group apart from that in insulin dose ($p = 0.001$).

	BRITTLE DIABETIC PATIENTS (N=6)	STABLE DIABETIC PATIENTS (N=7)	NORMAL CONTROL SUBJECTS N=6)
Maximum specific binding to adipocytes (%)	1.78±0.18	2.57±0.36	2.81±0.18
Insulin concentration for half maximal displacement (pmol/l)	206±42	122±13	113±10
Basal lipogenesis (pmoles per 10cm^2 per 90 min)	75±14	61±5	68±13
Maximal lipogenesis (pmoles per 10 cm^2 per 90 min)	181±30	187±37	173±29
Half-maximal stimulation of lipogenesis (pmol/l)	34±4	15±3	13±2
Total insulin degradation at 60 min (%)	1.1±0.1	1.4±0.1	1.2±0.1

TABLE 7.2 Adipocyte insulin binding, insulin sensitivity and insulin degradation in the non-diabetic group, and in the stable and brittle diabetic groups.

	SUBJECT 1		SUBJECT 2		SUBJECT 3	
	Initial	Repeat	Initial	Repeat	Initial	Repeat
Insulin therapy	I.M.	I.P.	I.M.	I.V.	I.V.	I.P.
HbA_1 %	12.2	9.1	18.2	10.0	9.2	8.5
Insulin dose U/Kg/day	1.64	0.57	2.59	0.82	2.13	0.90
Fasting P.F.I. mU/l	10.1	20.4	14.0	32.2	36	30.6
Max specific binding %	1.15	1.90	1.75	1.65	1.85	1.72
Half-max displ pmol/l	375	110	125	106	200	120
Basal L.G. pmol/90 min	74	89	59	46	87	55
Max L.G. pmol/90 min	202	169	107	92	246	143
Half-max stim pmol/l	46	12	44	6	32	23

TABLE 7.3 Individual adipocyte insulin binding and adipocyte insulin action data and clinical data upon the three brittle diabetic subjects re-studied after a period of stable diabetic control.
P.F.I. - plasma free insulin; L.G. - lipogenesis; max - maximum; displ - displacement

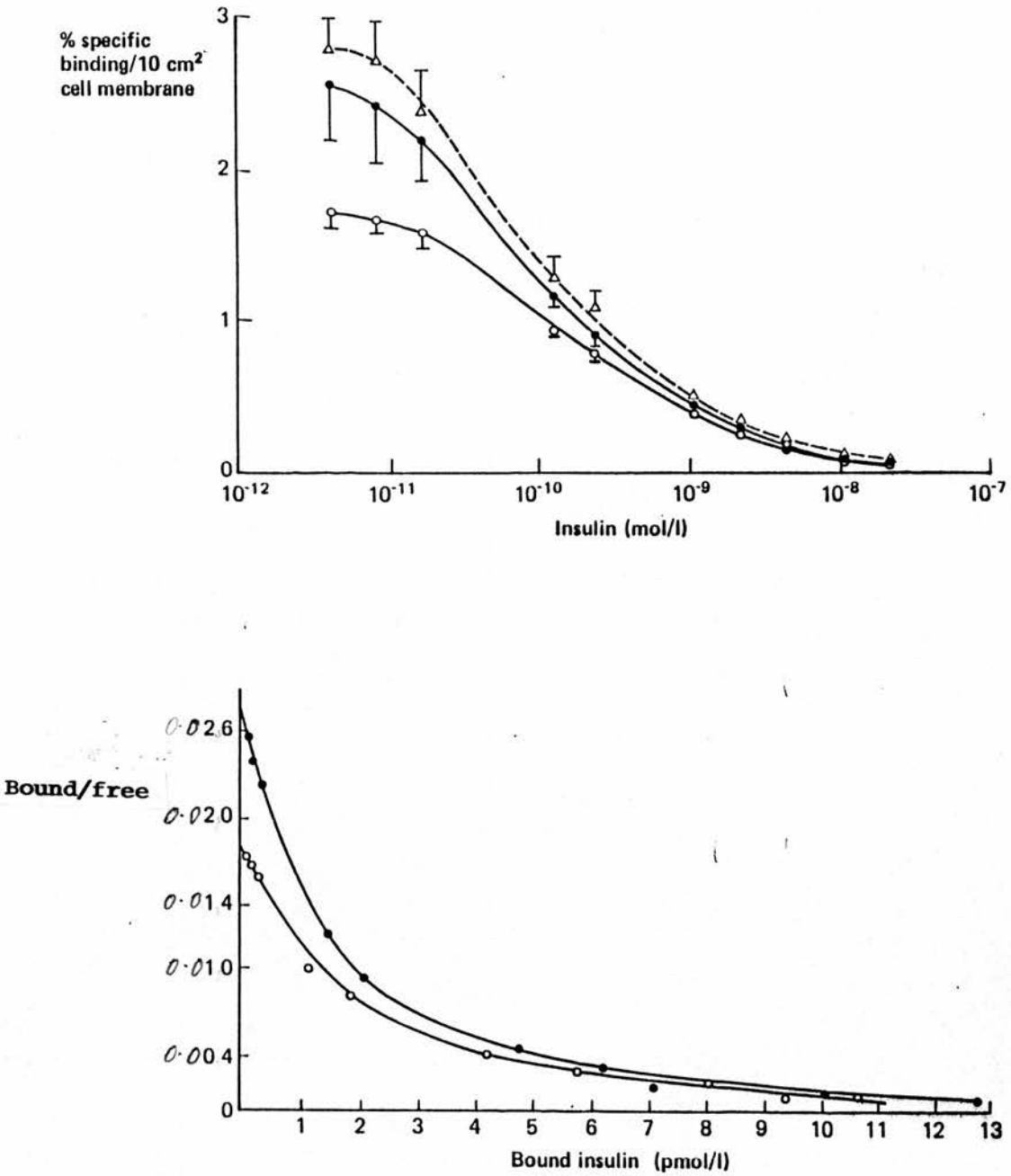

FIGURE 7.1 Specific insulin binding to adipocytes shown as binding displacement curves (top) and as Scatchard plots (bottom).

Brittle diabetic (o—o), stable diabetic (●—●) and non-diabetic (Δ--Δ) subjects.

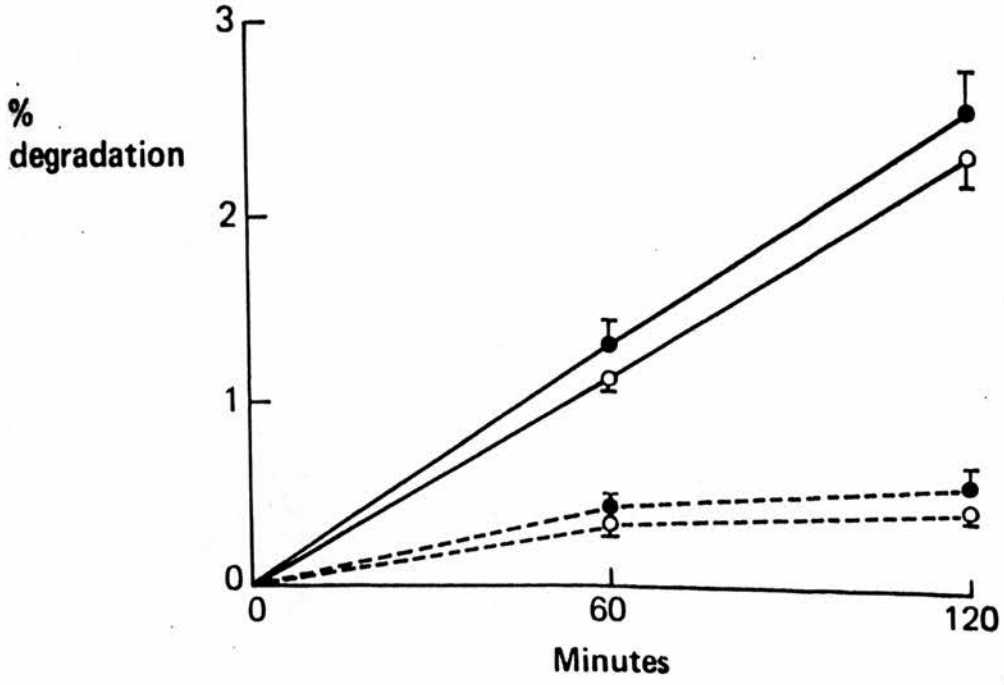

FIGURE 7.2 Degradation of tracer insulin in the presence of adipocytes from brittle diabetic (o——o) and stable diabetic subjects (•——•):

——— in the presence of tracer insulin alone.

------- in the presence of 10 μmol/l unlabelled insulin.

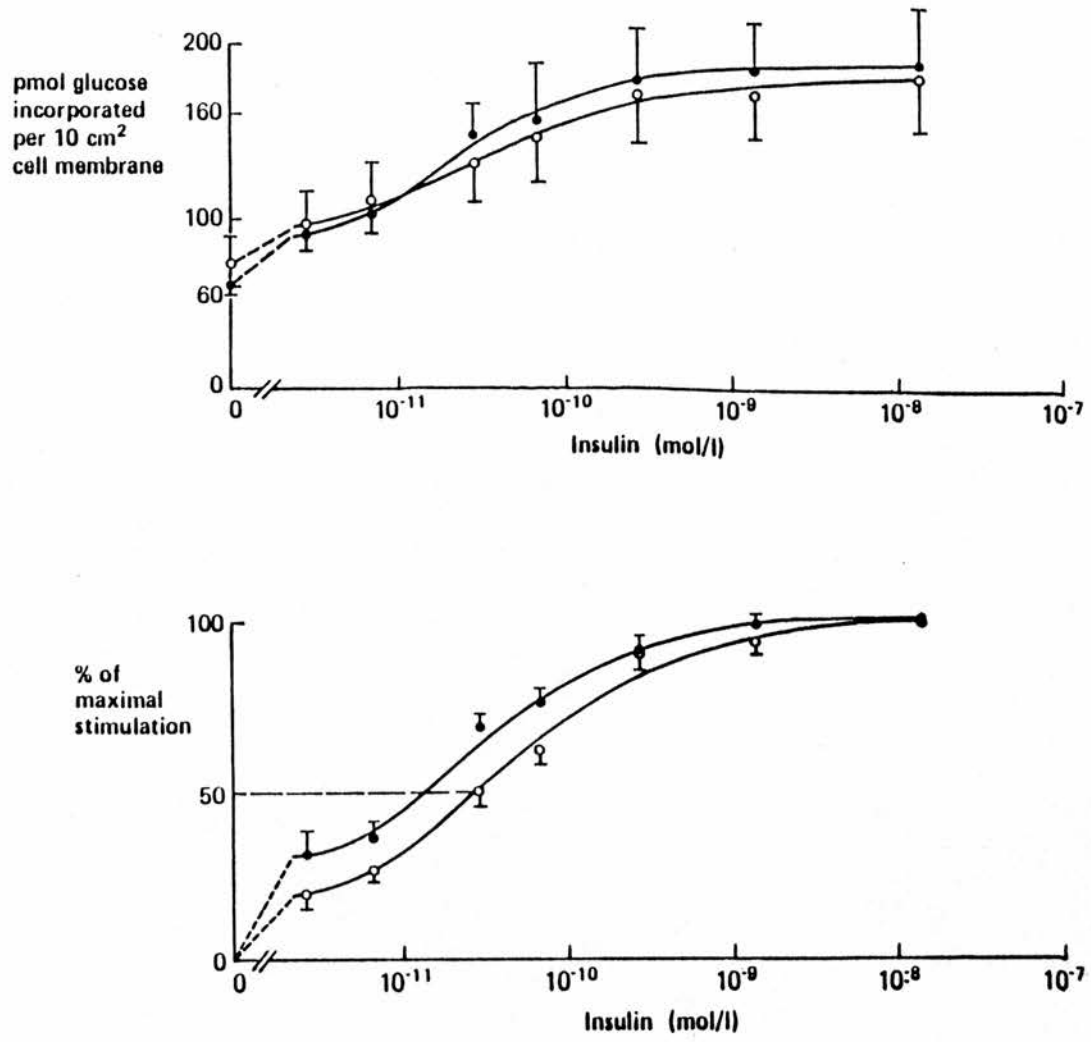

FIGURE 7.3 Insulin stimulation of lipogenesis shown as absolute rates (top) and as percentage stimulation (bottom). Dotted line indicates 50% stimulation.

Brittle (o—o) and stable (●——●) diabetics.

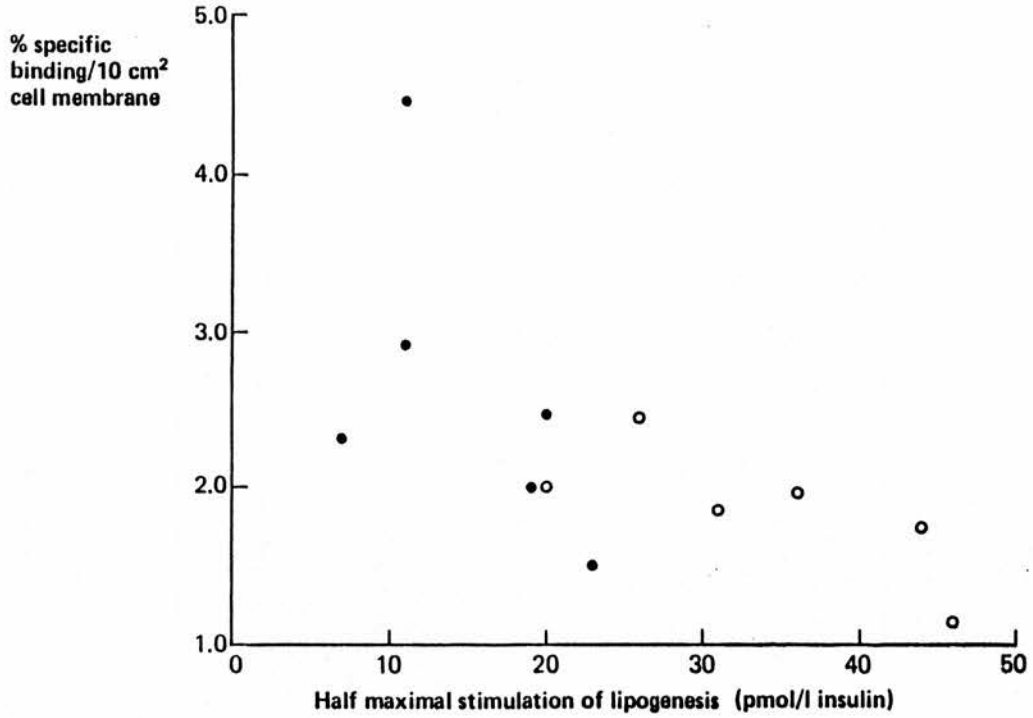

FIGURE 7.4 Relationship between maximum specific insulin binding and insulin concentration required for half-maximal stimulation of lipogenesis in adipocytes from brittle (o) and stable (•) diabetic subjects (R_s = 0.78; $p<0.01$).

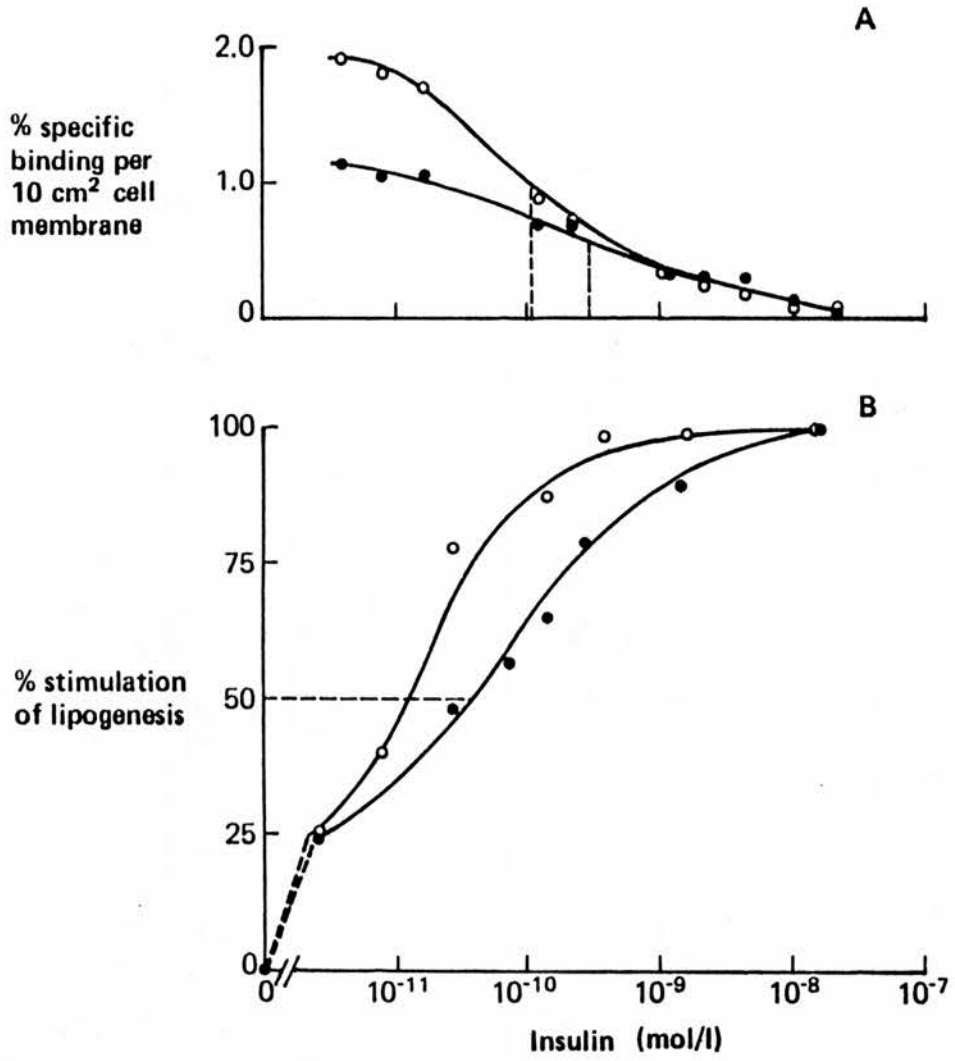

FIGURE 7.5 Specific insulin binding (top) and percentage stimulation of lipogenesis (bottom) in one brittle diabetic subject (Subject one in Table 7.3) during intramuscular insulin therapy (●——●) and after 9 months of intraperitoneal insulin administration (o——o). Dotted lines represent half-maximal displacement of insulin (top) and 50% maximal stimulation (bottom).

DISCUSSION

The adipocyte insulin binding in the stable diabetic group was not significantly different from that of the control group of non-diabetic subjects. This is at variance with Pedersen's finding of decreased adipocyte insulin binding in a group of stable insulin-dependent diabetic patients (Pedersen & Hjollund 1982). It is possible that the discrepancy in insulin binding data is a consequence of the selection criteria used in the two studies, poorly controlled stable diabetic subjects being specifically chosen as controls for the brittle diabetic subjects in the present study. Relative hypoinsulinaemia in these subjects could account for the differing results, and indeed the mean fasting plasma free insulin was only 50% of that reported by Pedersen. In the present study, basal and maximal rates of lipogenesis and the insulin sensitivity of lipogenesis were normal in the stable diabetic group. Normal adipocyte sensitivity to insulin stimulation of lipogenesis has been a consistant finding in studies of stable insulin-dependent diabetes (Pedersen & Hjollund 1982, Yki-Jarvinen *et al* 1984) although the sensitivity of adipocyte glucose transport has been reported to be normal in some studies (Yki-Jarvinen *et al* 1984) and diminished in others (Pedersen & Hjollund 1982). Yki-Jarvinen and colleagues (1984) observed normal basal and maximal rates of lipogenesis and glucose transport in adipocytes from moderately well controlled insulin-dependent diabetic subjects. Pedersen and Hjollund (1982) observed normal basal and maximal rates of glucose transport but decreased rates of lipogenesis in an apparantly similar study.

It cannot be certain that the diet, insulin therapy and metabolic control of the diabetic groups reported in different studies are strictly comparable. Improvement in glycaemic control in insulin-dependent diabetic subjects may be associated with an increase in insulin sensitivity *in vivo* (Beck-Nielsen *et al* 1984). In the present study, the stable diabetic subjects were more obese than the normal control subjects, but this factor would have tended to create rather than obviate differences in insulin binding and action. The small difference in age between the two groups would not be expected to influence the results, although studies of much older

subjects have demonstrated an effect of age upon adipocyte insulin sensitivity (Bolinder *et al* 1983, Fink *et al* 1983). The low-carbohydrate diets of the diabetic subjects are representative of the dietary intake of the majority of insulin-dependent diabetics at the present time. The effect of a difference in habitual calorie intake is unknown, and the absolute differences in carbohydrate and fat intake are small compared to those shown to be able to influence insulin binding (Ward *et al* 1982, Pedersen *et al* 1982b, Hjollund *et al* 1983a).

The brittle diabetic subjects were observed to have normal adipocyte receptor number but reduced adipocyte insulin receptor affinity and the reduction in affinity was reflected in resistance to insulin stimulation of lipogenesis *in vitro*. The insulin dosage in the brittle group was more than double that of the stable diabetic group and the possibility of receptor down-regulation by insulin must be considered. There was, however, no relationship between insulin dose and maximum insulin binding within either group. Similarly there was no relationship between insulin dose and half-maximal stimulation of lipogenesis. Fasting plasma free insulin levels were markedly higher in those brittle diabetic patients receiving insulin by intravenous infusion (74 $\pm$ 24 mU/l) rather than by intermittent intramuscular injection (8.7 $\pm$ 3.5 mU/l), but this bore no relationship to the extent of the decrease in insulin binding or insulin sensitivity. Furthermore, the mean fasting plasma free insulin level was higher in the stable group than in the brittle subjects receiving intramuscular insulin. Dietary factors have been shown to influence cellular insulin binding (Ward *et al* 1982, Hjollund *et al* 1983a) but the dietary differences between the brittle diabetic group and the stable diabetic subjects were relatively small and would not have been expected to affect insulin binding. The two diabetic groups were well matched with respect to age and body weight.

Previous studies *in vivo* and *in vitro* have suggested that short-term down-regulation of insulin receptors can be mediated by reduction in cell surface receptor number (Marshall & Olefsky 1980, Pezzino *et al* 1980). Studies of adipocyte insulin binding in various

clinical states of insulin resistance have also suggested that reduction in adipocyte insulin binding is most often a result of reduced receptor number (Olefsky 1976, Olefsky et al 1982, Pedersen & Hjollund 1982). Short term changes in insulin receptor affinity have been recorded after acute changes in food intake (Bar et al 1976, Beck-Nielsen & Pedersen 1978, Livingston & Moxley 1982) and after hydrocortisone (Yasuda & Kitabchi 1980) or growth hormone administration (Bratusch-Marrain et al 1982). Examples of chronic reduction in receptor affinity are rare, having been demonstrated during high fat diets (Beck-Nielsen et al 1978), in one study of obese subjects (Pedersen et al 1981c) and during glucocorticoid administration in rats (Kahn et al 1978). The reduction of receptor affinity observed in the group of brittle diabetics is therefore of particular interest. The cause of this change in receptor affinity is uncertain. No abnormalities in serum glucagon, growth hormone, prolactin nor catecholamines have been observed in brittle diabetes and although morning cortisol levels have been noted to be somewhat elevated, mean 24 hour serum cortisol levels in the same subjects were unremarkable (Massi-Benedetti et al 1984). Plasma non-esterified fatty acid and total ketone body levels were observed to be variable but not consistently high (Schade et al 1980). The syndrome of brittle diabetes is intimately related to the administration of extremely variable insulin doses over a long period of time. As insulin itself appears to be a major factor regulating the insulin receptor, the observation of an unusual adipocyte insulin receptor status is perhaps not surprising.

It has been suggested that increased degradation of insulin in the subcutaneous tissues may account for the poor response to subcutaneous insulin characteristic of brittle diabetic patients (Berger et al 1979, Gill et al 1984). The studies on insulin degradation presented here demonstrate that the adipocytes themselves are not responsible for any such abnormal degradation, although other subcutaneous components could be involved in insulin degradation. The similarity in insulin degradation in the presence of adipocytes from each of the groups also eliminates this as a possible cause of the observed low insulin binding to the cells of the brittle diabetic patients.

Although intramuscular insulin is sometimes effective in achieving adequate plasma free insulin levels and reasonable metabolic control in the short term (Pickup et al 1981 & 1983), this is often not so and long term intravenous insulin may be required (Berger et al 1979). Both of these routes induce hyperinsulinaemia in the peripheral circulation, a factor which can be avoided at least in part by peritoneal delivery of insulin (Schade et al 1980, Pozza et al 1983, Husband et al 1984). In one brittle patient restudied after a 9 month period of satisfactory metabolic control on intraperitoneal insulin therapy, adipocyte insulin binding reverted towards the mean of the stable diabetic group secondary to an increase in receptor affinity. In the patients restudied during treatment with continuous intraperitoneal and intravenous insulin infusion, insulin receptor affinity also appeared to increase, as evidenced by decreased insulin concentrations required for half-maximum displacement of tracer insulin. Despite this, maximum specific binding was not affected, suggesting a simultaneously decreased receptor number. In all three subjects, a decrease in the insulin concentration necessary to achieve half-maximal stimulation was observed. Conventionally, this may be taken to indicate increased cellular insulin sensitivity (Kahn 1978). However, a fall was observed in the maximally insulin stimulated rate of adipocyte lipogenesis for all three subjects during the period of stable control. This change during the period of stable diabetic control is difficult to explain, and suggests the development of a post-binding defect of insulin stimulation of lipogenesis. The reversal of the abnormalities of binding affinity and insulin sensitivity of brittle diabetes brought about by this change in route of administration and dose of insulin indicates that the reduced receptor affinity and adipocyte insulin resistance are unlikely to be primary changes. Once present, however, these changes are likely to exacerbate the clinical problems. Further study is required to define the precise aspect of the abnormal metabolic state which induces the cellular insulin resistance in brittle diabetes.

In conclusion, reduced adipocyte insulin receptor affinity and reduced adipocyte insulin sensitivity have been observed in brittle diabetes. These abnormalities were observed during treatment with high doses of insulin administered intramuscularly or intravenously.

No direct relationship of the tissue abnormalities to insulin dose or to plasma free insulin levels could be established. The reversal of the abnormalities during periods of stable diabetic control achieved by continuous intra-peritoneal or intravenous insulin infusion suggests that changes in tissue insulin sensitivity may not be the primary cause of the syndrome.

CHAPTER EIGHT

GENERAL DISCUSSION

CONTENTS OF CHAPTER EIGHT

METHODOLOGY

Results of *in vivo* and *in vitro* studies of insulin sensitivity and responsiveness depend heavily upon the accuracy, precision and relevance of the techniques used. Adipose tissue from the gluteal region was used for all the clinical studies as it has been demonstrated that adipocytes from different anatomical sites do not display identical metabolic characteristics (Bolinder *et al* 1983b). There is no evidence to suggest that the subcutaneous tissue of any one region is more relevant to whole body metabolism than that of other regions. The selection of the gluteal region is of practical importance, adipose tissue biopsy being possible at this site even in lean subjects. The potential effects of lignocaine upon adipocyte cell membrane function must be considered, even though it was injected intradermally rather than subcutaneously. However, multiple injections of lignocaine into a biopsy sample was found not to affect adipocyte function (Pedersen 1982).

The adipocyte insulin binding assay used in the present studies had a high precision and was associated with low insulin degradation and a lower non-specific binding than previously reported (Olefsky 1976, Harewood *et al* 1982, Pedersen 1983). Accuracy of the assay was not formally determined, but Hjollund and colleagues (1983) found an identical assay to be highly reproducible. Specific insulin binding was measured at true steady state and thus represented a measure of physiologically active insulin receptors. All insulin binding and action data was related to adipocyte surface area, rather than cell number. Adipocytes have a very thin rim of cytoplasm surrounding the lipid store which occupies most of the cell volume. The surface area may be anticipated to be more closely related to acute metabolic changes than cell volume. Adipocytes from different individuals vary markedly in size, and this influences any parameter expressed as a function of cell number. Thus, the adipocytes of normal young males and females appear to have different insulin binding capacities and different basal and maximal rates of glucose transport and metabolism if cell number, rather than surface area is used as denominator (Pedersen *et al* 1982a). In contrast to studies comparing male and

female or lean and obese subjects, mean cell diameter did not differ between control and study groups in any of the present studies, and hence expression of data per cell number, per cell volume or per cell surface area would not affect the findings.

Insulin action upon isolated adipocytes was assessed by measuring rates of glucose incorporation into total lipids. Glucose uptake into the cell, conversion to glycerol phosphate and esterification is therefore assessed. It is likely that glucose uptake is the rate limiting step (Pedersen et al 1982a). When initial rates of glucose transport were measured directly, the results agreed with those of lipogenesis studies (Chapter 6). It may not be assumed that the insulin sensitivity of any one metabolic pathway reflects that of others. Glucose oxidation and lipolysis appear to be differentially affected in insulin-dependent diabetes (Pedersen & Hjollund 1982). However, storage of glucose as lipid rather than inhibition of lipolysis is relevant to the processes involved in the assessment of insulin sensitivity in vivo (lipogenesis and glycogen synthesis). It would have been of interest to examine all aspects of adipocyte insulin action in the present studies, but it was not technically feasible to perform singlehandedly more than one insulin action assay in addition to the binding studies upon each biopsy sample.

The monocyte insulin binding assay compared favourably with other published assays by the criteria of precision, tracer insulin degradation, steady state binding and non-specific binding. Data on accuracy of monocyte binding assays have not previously been reported. All assays of blood cell insulin binding are performed at sub-physiological temperatures, tracer insulin degradation being excessively rapid at 37°C, and this factor could distance the in vitro observations from in vivo cellular receptor status. The effect of assay temperature per se does not correct for the lack of correlation with adipocyte insulin binding, Pedersen and Hjollund (1982) having compared insulin binding to monocytes at 15°C and adipocytes at both 15 and 37°C. Unlike adipocytes, monocytes bind insulin with a pH optimum of 8.0 and all reported studies have been carried out at this pH. Although this factor is unlikely to distort

the results of comparative studies of disease states, it does imply a lack of direct physiological relevance for monocyte insulin binding. The term "monocyte insulin binding" is traditionally used to describe the insulin binding properties of a mixed mononuclear cell suspension. Lymphocytes comprise approximately 80% of the cell number but account for less than 20% of the specific binding activity in such a suspension. Large changes in lymphocyte number or properties would thus be required to influence the capacity for insulin binding observed in the mixed cell suspensions. Provided that the method for determining the number of monocytes is accurate, then it may be preferable to accept the implications of measurements upon the mixed cell population rather than subject monocytes to lengthy and metabolically perturbing separation techniques.

Several methods have been used for the assessment of insulin sensitivity in vivo. The intravenous insulin tolerance test and the oral glucose / subcutaneous insulin test both fail to establish steady state insulin action, and secretion of counter-regulatory hormones complicates interpretation of the former test. The euglycaemic hyperinsulinaemic clamp technique allows assessment of glucose disposal rates during steady state insulin action. The insulin infusion rate chosen for the present studies generates peripheral plasma insulin levels equivalent to normal post-prandial levels. It has been shown that hepatic glucose output is decreased by more than 90% under the clamp conditions in both normal and insulin resistant subjects (DeFronzo et al 1983a). Under conditions of euglycaemic hyperinsulinaemia, glucose uptake by the peripheral tissues is favoured, only 5 to 10% of an intravenously administered glucose load being taken up by the splanchnic bed (DeFronzo et al 1978b). The rate of glucose infusion thus reflects glucose uptake by muscle and adipose tissue, muscle accounting for approximately 85% of total glucose disposal (DeFronzo et al 1981a). Valid comparison of insulin mediated glucose disposal rates between different subject groups depends heavily upon the steady state blood glucose levels and upon the variablility of blood glucose during the clamp period. Both of these factors were satisfactory in the present studies (Chapters 2 & 4).

The ability of the euglycaemic clamp technique to distinguish between marked degrees of insulin resistance at physiological levels of hyperinsulinaemia is limited by the rate of non-insulin-dependent glucose utilisation (approximately 1.5 mg/kg/min in normal subjects). The glucose/insulin infusion technique avoids this difficulty, the parameter measured - steady state blood glucose concentration - having no fixed limits. In normal subjects, endogenous insulin secretion is suppressed during the test. Although insulin secretion occurs in subjects with raised steady state blood levels and peripheral tissue glucose uptake is increased by a mass action effect (DeFronzo et al 1983a), this should not impair distinction between degrees of insulin resistance. It has been demonstrated that the results of glucose/ insulin infusion and euglycaemic clamp correlate well (Heine et al 1982) and hence it is unlikely that the magnitude of any mass action effect could change the ranking of the cirrhotic subjects with respect to degree of insulin resistance.

RELATIONSHIP BETWEEN DIFFERENT TISSUES

The overall metabolic response of the body depends upon the integrated function of liver, muscle and adipose tissue. The metabolic behaviour of each tissue is regulated both by hormonal influence and substrate supply (Randle et al 1966). It is therefore not surprising that one tissue should display hormonal responses which are not reflected by other tissues. The disparate behaviour of liver compared to peripheral tissues in hyperthyroidism (Chapter 6) is an example of this and it cannot be assumed that one tissue regulates any one step in cellular insulin action in precisely the same manner as another tissue, particularly if hormonal and metabolic environments differ markedly. The widespread assumption that circulating monocytes reflect the insulin receptor status of insulin sensitive tissues thus appears insubstantial on theoretical grounds alone. It is noteworthy that only one relevant study is quoted to support this hypothesis (Olefsky 1976). The hypothesis was indirectly supported by consistant observations of low insulin binding to all cell types in obesity, although the pitfalls in extrapolating from these observations were discussed at the start of the era of clinical

studies of blood cell insulin binding (Olefsky 1976). The data of Pedersen and colleagues (1982a, Pedersen & Hjollund 1982) and the data presented in Chapter 3 demonstrate that the monocyte cannot be accepted as an accurate and convenient mirror of the insulin receptor status of adipose tissue.

The assumed relevance to carbohydrate metabolism of blood cell insulin receptor status has inhibited study of the physiological function of insulin receptors on these cells. Several isolated findings are of interest in this respect. Insulin receptors on non-cytotoxic T-lymphocytes are not expressed unless the cells are exposed to specific antigenic stimulation (Braciale et al 1982). Insulin has been shown to influence the cell membrane fluidity of erythrocytes (Bryszewska & Leyko 1983), and studies of the physiological relevance and mechanism of this finding will be of interest.

The two major peripheral insulin sensitive tissues, muscle and fat are exposed to the same hormonal and metabolic influences. In hepatic cirrhosis, obesity and insulin-dependent diabetes the low adipocyte insulin receptor number and adipocyte insensitivity to insulin appear to parallel the in vivo observations on muscle insulin sensitivity, as measured by the euglycaemic clamp technique (Iversen et al 1984, Taylor et al 1984a, Olefsky et al 1982a, DeFronzo et al 1982, Pedersen & Hjollund 1982). In hyperthyroidism, the sensitivity of both adipocytes in vitro and muscle in vivo were found to be normal (McCulloch et al 1983a, Taylor et al 1984c). However, it may not be inferred that adipocytes and myocytes always display similar changes in insulin sensitivity in all clinical states. The observation of differential sensitivity of antilipolysis and glucose metabolism even in the same cell type (Pedersen & Hjollund 1982) does suggest that general comparison of the insulin sensitivity of cells of disparate metabolic function are unlikely to be well founded. The normal adipocyte insulin sensitivity but impaired myocyte insulin sensitivity in chronic renal failure (DeFronzo & Smith 1982, Chapter 5) may represent one example of different responses of the two major peripheral tissues, although the possibility of circulating inhibitors of insulin action in this condition (Dzurik et al 1969,

Lockwood & McCaleb 1983) prevents direct comparability of *in vivo* and *in vitro* studies. A more precise understanding of the relative behaviour of adipocyte and myocytes must await the performance of simultaneous *in vitro* studies upon the two cell types.

INTERPRETATION OF GLUCOSE TOLERANCE TEST DATA

The data presented in Chapters 4, 5 and 6 demonstrate a notable lack of relationship between glucose tolerance and adipocyte insulin sensitivity. In cirrhotic subjects, no relationship between *in vivo* insulin sensitivity and glucose tolerance could be demonstrated. These findings suggest that tolerance to oral glucose depends upon peripheral tissue insulin sensitivity to only a small extent. Direct measurement of splanchnic glucose balance during the 75 gram oral glucose tolerance test in normal subjects has shown that 50 - 60 % of the administered glucose is taken up by splanchnic tissues (predominantly liver) (Waldhausl *et al* 1983), whereas only 10 % of an intravenously administered glucose load is taken up by the liver (DeFronzo *et al* 1978b). This difference is not an effect of glucose delivery to the liver via the portal vein, as approximately 90 % of orally administered glucose appears in the systemic circulation (Radziuk *et al* 1978). Hollenbeck and colleagues (1984) have estimated that peripheral tissue responses to insulin following oral glucose account for only one third of the observed overall response. Extrapolation of these studies to any pathological state is complicated by variable hepatic insulin extraction and hence variable peripheral plasma insulin levels (Bratusch-Marrain *et al* 1984). Decreased hepatic insulin extraction in cirrhosis (Greco *et al* 1979, Riggio *et al* 1982) may be expected to allow higher peripheral insulin levels and hence a relatively greater effect upon peripheral tissues were it not for the presence of peripheral tissue insulin resistance. Porto-systemic shunting of blood *per se* does not appear to have a marked influence upon glucose tolerance in cirrhosis (Johnston *et al* 1978).

Treatment of chronic renal failure by C.A.P.D. brings about a decrease in fasting blood glucose, most likely as a secondary

consequence of reduction in hepatic glucose output (DeFronzo et al 1983a). Fasting serum insulin levels remained unchanged after C.A.P.D. implying that the liver had become more insulin sensitive. Despite this, glucose tolerance remained abnormal, suggesting impaired sensitivity of both liver and peripheral tissues at higher insulin concentrations.

Hyperthyroidism provides an interesting model for the study of hepatic function after an oral glucose load, peripheral insulin sensitivity being normal in this condition (McCulloch et al 1983a). The hyperglycaemia observed is therefore likely to be a direct consequence of decreased hepatic glucose uptake. In hyperthyroid subjects, high peak but almost normal two hour blood glucose concentrations were observed. This could represent an effect of peripheral tissues responding to the combination of hyperglycaemia and normal plasma insulin levels, perhaps together with a greater hepatic response at high plasma insulin levels. Study of the tissue distribution of an oral glucose load in hyperthyroid subjects could distinguish between these possibilities.

RELATIONSHIP BETWEEN RECEPTOR BINDING AND INSULIN ACTION IN ADIPOCYTES

The original descriptions of low insulin receptor number on monocytes in insulin resistant states led to the concept that decreased insulin receptor number was a major cause of cellular insensitivity to insulin action (Bar et al 1976, Olefsky & Kolterman 1982, Roth & Taylor 1982). The tendency during the 1970's to introduce all papers upon blood cell insulin binding with the comment that the first step in the action of peptide hormones consisted of interaction of the hormone with specific cell surface receptors, suggests an underlying belief that the first step was also the major regulatory step. Recognition of states of cellular insulin resistance characterised by normal insulin receptor number and affinity led to the concept of rate-limiting steps in the intracellular pathways of insulin action (Bollinder et al 1982). The discovery of the protein kinase activity of the insulin receptor (Roth & Cassel 1983) and the

hypothesis of a cascade of phosphorylation / dephosphorylation reactions conveying the insulin signal within the cell (Denton et al 1981) has indicated a mechanism for the linkage between receptor and intracellular action, but the exact nature of this linkage remains obscure.

Insulin receptor internalisation normally occurs simultaneously with insulin action (Olefsky et al 1982b). Receptor downregulation by insulin depends upon regulation of receptor internalisation and recycling to the cell membrane. It has been postulated that post-binding defects in insulin action may also bring about failure of downregulation of insulin receptors (Caro & Amatruda 1980, Misbin et al 1983). This hypothesis could explain the anomalous occurrence of normal insulin receptor number but impaired cellular insulin action in the presence of hyperinsulinaemia (Bolinder et al 1982). In subjects with cryptogenic and primary biliary cirrhosis, adipocyte insulin sensitivity was found to be impaired, but adipocyte insulin receptor binding was only minimally decreased (Chapter 4).

The combination of decreased insulin receptor number and normal cellular insulin sensitivity has only been observed in hyperthyroidism (Chapter 6). Thyroid hormones, or some secondary effect of excessive thyroid hormone levels, must act upon the processes regulating receptor number, in addition to inducing compensatory changes so that cellular insulin sensitivity remains normal. These observations suggest that each of the steps of cellular insulin action, receptor and post-binding, may be amenable to separate regulation. Overall cellular insulin action must be regarded as a chain of events, each link of which may be capable of being regulated. The insulin receptor is but one link in this chain.

CONTROL OF CELLULAR INSULIN ACTION

Insulin was the first factor recognised to regulate insulin receptor number (Gavin et al 1974). The concurrence of hyperinsulinaemia and insulin resistance was postulated to be a reflection of the role of hyperinsulinaemia in the genesis of

cellular insulin resistance (Bar *et al* 1976). The proposed model was based upon a circular argument, involving hyperinsulinaemia, decreased insulin receptor number, decreased insulin action, hyperglycaemia and hence hyperinsulinaemia. The initiating factors have never been identified. Short-term insulin infusion *in vivo* does indeed induce a degree of adipocyte insulin resistance (Mandarino *et al* 1984), but glucose administration orally or intravenously to normal subjects is associated with a pronounced increase in adipocyte insulin sensitivity (Arner *et al* 1983, Livingston *et al* 1984). Eight day infusion of insulin into rats has been reported to increase both *in vivo* insulin sensitivity and *in vitro* adipocyte insulin sensitivity (Trimble *et al* 1984). It is apparent that insulin is only one regulator of cellular insulin sensitivity, and that the acute and chronic effects of other hormones and of substrate supply upon individual tissues must be evaluated.

The integrated effects of insulin and metabolic state are of relevance to insulin-dependent diabetes. Poor diabetic control is associated with insulin resistance both *in vivo* and in isolated adipocytes, whereas during periods of optimal control normal insulin sensitivity is observed (Pedersen & Hjollund 1982, Lonnroth *et al* 1983b, Hjollund *et al* 1983a, Reevers *et al* 1984). Insulin resistance and poor control was observed in brittle diabetes (Chapter 7). Restoration of stable control on a lower daily insulin dose brought about an increase in adipocyte insulin sensitivity. This was achieved by alteration not only of total insulin dosage but also of route and temporal pattern of insulin delivery. In the small number of subjects restudied, the difference between administration of insulin intraperitoneally or intravenously in terms of adipocyte function was not great. The constant rate of insulin infusion may have been expected to have a lesser biological effect than a regimen employing intermittent insulin boluses (Matthews *et al* 1984).

PATHOPHYSIOLOGY OF INSULIN RESISTANCE IN METABOLIC DISEASE

The *in vivo* defect in insulin sensitivity identified in subjects with hepatic cirrhosis was found to be reflected in adipocyte behaviour *in vitro*. However, the observation of marked differences between the aetiological sub-groups of cirrhotic subjects complicates interpretation of data. Both insulin receptor and post-binding defects in insulin action were evident in adipocytes from subjects with alcoholic cirrhosis. Insulin receptor number was only slightly decreased but insulin sensitivity was markedly impaired in adipocytes from subjects with primary biliary and cryptogenic cirrhosis. The severity of impairment of liver function was similar in all three subgroups as judged by the usual clinical and metabolic criteria. The effect of alcohol, either directly or secondarily via hormonal or metabolic effects, is most relevant to these findings. Chronic and excessive alcohol intake has been reported to be associated with mild elevation of plasma cortisol (Margraf *et al* 1967) and catecholamines (Ogata *et al* 1971) and with impaired regulation of growth hormone secretion (Chalmers *et al* 1977). Episodic hypoglycaemia and normoglycaemic ketoacidosis may be frequent and underdiagnosed in chronic alcoholics (Marks 1978, Palmer 1983). Comparison of adipocytes from age, sex, weight and diet matched groups of non-cirrhotic and cirrhotic alcoholics could identify the separate effects of cirrhosis and chronic alcohol intake.

The insulin action data have been discussed in terms of insulin sensitivity of adipocytes as represented by the insulin concentration necessary to achieve half-maximal stimulation (Kahn 1978). Responsiveness, defined as the absolute increase above basal rates, was found to be paradoxically greater in the primary biliary and cryptogenic subgroups. The implications of this are uncertain particularly as exaggerated rates of lipogensis would not occur at physiological insulin levels. Maximal rates of lipogenesis as measured *in vitro* must be regarded as a theoretical concept. If it is assumed that the differences between the subgroups in subject matching are relatively unimportant, then the parameter of insulin action which agrees most closely with data from the *in vivo* studies is that of sensitivity. The insulin concentration achieving half-

maximal stimulation of lipogenesis also appeared more relevant to *in vivo* insulin sensitivity in hyperthyroid subjects, uraemic subjects on C.A.P.D., and brittle diabetic subjects after restoration of stable control. This interpretation assumes that the insulin sensitivity of muscle and fat are similarly regulated *in vivo*. At the present time, the conventional interpretation of insulin action data cannot be rejected in favour of a more satisfactory system.

The role of elevated serum insulin levels in bringing about adipocyte insulin receptor downregulation in cirrhosis must be considered in view of the correlation between serum insulin and adipocyte insulin binding. However, it is possible that a decrease in insulin receptor number induced the hyperinsulinaemia. The lack of relationship between either mean serum insulin or *in vivo* insulin sensitivity and insulin receptor status of the cirrhotic subgroups suggests that post-binding abnormalities of insulin action play the more important role in peripheral tissue insulin insensitivity in cirrhosis. The isolation and partial chracterisation of a circulating inhibitor of insulin action (Dzurikova *et al* 1974) raises interesting questions about the influence of such a substance upon the post-binding pathways of insulin action.

In chronic renal failure, *in vivo* studies have demonstrated clearly that insulin action upon muscle is decreased (Westervelt 1969, DeFronzo & Smith 1982). The present observation of normal *in vitro* adipocyte insulin sensitivity may be interpreted as supporting the concept of a circulating inhibitor of insulin action (Dzurik *et al* 1969, Lockwood & McCaleb 1983). Sudden removal of this inhibitor could account for the slightly greater *in vitro* sensitivity of adipocytes from uraemic subjects. If C.A.P.D. decreased circulating levels of any such inhibitor, then the moderate right shift of dose response curve for stimulation of lipogensis (Figure 5) could be explained, compensation for the inhibitory influence no longer being induced. The probable greater sensitivity of the liver to insulin in the suppression of fasting hepatic glucose output could be speculated to be an effect of the removal by dialysis of an inhibitor of insulin action. If the *in vitro* binding studies are assumed to reflect *in vivo* insulin receptor status, then within the physiological range of

insulin concentrations the increase in affinity would compensate for the decrease in receptor number. Hence, any impairment of insulin sensitivity would be due entirely to post-binding defects. This conclusion may be supported by data from multi-step hyper-insulinaemic, euglycaemic clamp studies, and the observed phenomena could be explained without the need to adduce abnormalities of overall insulin receptor function. However, the direct relevance of adipocyte insulin receptor studies will remain uncertain until the proposed inhibitor of insulin action is characterised. If an inhibitor competitively interferes with insulin binding as reported by Milutinovic (1983), then measurement of binding *in vitro* may be expected to be associated with increased affinity.

The finding of normal insulin sensitivity in adipocytes from hyperthyroid subjects is complemented by the observation of normal peripheral tissue insulin sensitivity *in vivo* (McCulloch *et al* 1983a). There can be little doubt that the abnormalities of carbohydrate metabolism in hyperthyroidism are mainly a result of altered hepatic function. A low adipocyte insulin receptor number suggests an effect of elevated thyroid hormone levels upon regulation of cellular insulin receptor number and also that such an effect can be wholly compensated by post-binding modulation of the insulin signal.

Decreased receptor affinity was demonstrated in adipocytes from brittle diabetic subjects, and this appeared to correlate with decreased adipocyte insulin sensitivity. Although there is no evidence to suggest any post-binding defect, it is not possible to declare that the intracellular pathways of insulin action are normal in adipocytes from these subjects. However, the data obtained are consistent with a receptor defect. It is possible that the abnormal pattern of insulin administration brings about the observed changes, although attempts to induce receptor defects in normal subjects by constant insulin infusion have been unsuccessful (Mandarino *et al* 1984), post-binding defects in insulin action being induced instead. This suggests that insulin could exert a primary effect upon intracellular mechanisms, and that observed receptor changes may be secondary to intrinsic cellular control mechanisms (Caro & Amatruda

1980, Misbin et al 1983). Although the abnormalities in the adipocytes of brittle diabetic subjects are of considerable interest, they appear unlikely to be directly related to the primary cause of the syndrome.

In summary, the results of these studies emphasise the importance of the intracellular steps of insulin action in determining the response of individual tissues in metabolic disease. The role of the insulin receptor as the initiator of a series of intracellular events is confirmed, and the need is clear for detailed study of the control of each post-binding step of insulin action.

APPENDICES

APPENDIX ONE: COMPOSITION OF BUFFERS

Collagenase buffer

NaCl	135 mmol/l
KCl	4.8 mmol/l
$MgSO_4$	1.7 mmol/l
$CaCl_2$	2.5 mmol/l
NaH_2PO_4	0.2 mmol/l
Na_2HPO_4	1.0 mmol/l
HEPES	10 mmol/l
Glucose	5 mmol/l
Human serum albumin	25 mg/ml
Crude collagenase	0.5 mg/ml

pH 7.42 at 37°C

Lipogenesis buffer

Used for washing adipocytes and for assays of glucose transport and lipogensis in adipocytes.

NaCl	135 mmol/l
KCl	4.8 mmol/l
$MgSO_4$	1.7 mmol/l
$CaCl_2$	2.5 mmol/l
NaH_2PO_4	0.2 mmol/l
Na_2HPO_4	1.0 mmol/l
HEPES	10 mmol/l
Human serum albumin	50 mg/ml

pH 7.42 at 37°C

Receptor buffer

Used for assay of insulin binding to adipocytes.

NaCl	135 mmol/l
KCl	4.8 mmol/l
$MgSO_4$	1.7 mmol/l
$CaCl_2$	2.5 mmol/l
NaH_2PO_4	0.2 mmol/l
Na_2HPO_4	1.0
HEPES	10 mmol/l
Glucose	0.5 mmol/l
Human serum albumin	50 mg/ml

pH 7.42 at 37°C

Monocyte binding buffer

NaCl	120 mmol/l
KCl	5.0 mmol/l
$MgSO_4$	1.2 mmol/l
Na acetate	15 mmol/l
EDTA	1.0 mmol/l
Bacitracin	0.5 mg/ml
Bovine serum albumin (Fraction V)	10 mg/ml

pH 8.0 at 20°C

APPENDIX TWO: LIST OF ABBREVIATIONS

A.M.P.	adenosine monophosphate
A.S.T.	aspartate transaminase
C.A.P.D.	continuous ambulatory peritoneal dialysis
cm	centimetre
C-peptide	connecting peptide
^{o}C	degrees centigrade
D-(U-^{14}C)	Dextro isomer uniformly labelled with carbon 14
E.D.T.A.	ethylenediamine tetra-acetic acid
g	unit gravity
HbA_1	glycosylated haemoglobin
^{125}I	iodine 125
I.B.W.	ideal body weight
I.R.I.	immunoreactive insulin
kg	kilogram
l	litre
LG	lipogenesis
max	maximum
mg	milligram
min	minute
ml	millilitre
mmol	millimole
mU	milliunit
µCi	microcurie
µm	micrometre
µl	microlitre
N.I.D.D.	non-insulin dependent diabetic
nmol	nanomole
N.S.	not significant
p	probability
P.B.C.	primary biliary cirrhosis
P.F.I.	plasma free insulin
pH	log hydrogen ion concentration
pmol	picomole
P.T.H.	parathyroid hormone
R	linear correlation coefficient
R_s	Spearman rank correlation coefficient
sec	second
vs	versus

APPENDIX THREE: SOURCE OF MATERIALS USED

Albumin, bovine Fraction V	Sigma Chemical Company Ltd, Poole, Dorset
Albumin, bovine >99% pure	Hoechst UK Ltd, Houndslow, Middlesex
Albumin, human >99% pure	Hoechst UK Ltd, Houndslow, Middlesex
Bacitracin	Sigma Chemical Company Ltd, Poole, Dorset
Collagenase, crude	P-L Biochemicals, Milwaukee, Wisconsin, U.S.A.
Collagenase, crude	Worthingron Biochemical Corp, Freehold, New Jersey, U.S.A.
Cover slide, plastic	Micropure Holland, Rijsenburg, Holland
Fenwal triple pack	Travenol Labs Ltd, Thetford, Norfolk
Ficoll, M.W. 400,000	Sigma Chemical Company Ltd, Poole, Dorset
Glucose, D-(U-^{14}C) S.A. 270 Ci/mol	Amersham International, Little Chalfont, Buckinhamshire
Haemaccel	Hoechst UK Ltd, Houndslow, Middlesex
Hepes	Sigma Chemical Company Ltd, Poole, Dorset
Hypaque, 65%	Sterling Research Labs, Surbiton upon Thames, Surrey
Infusaid pump	Infusaid Corporation, Norwood, Massachussetts, U.S.A.
Insulin, crystalline porcine	Novo Research Institute, Copenhagen, Denmark
Insulin, A_{14}-^{125}I, S.A. 210-240 μCi/ug	Novo Research Institute, Copenhagen, Denmark
Medium 199	Flow Laboratories, Irvine, Ayrshire
Naphthyl acetate	Sigma Chemical Company Ltd, Poole, Dorset
Nylon mesh, 20 denier tights	Marks & Spencer, Baker St, London
Pipe cleaners, Pathfinder	Robert Sinclair Ltd, Leeds
Pararosanilin hydrochloride	Sigma Chemical Company Ltd, Poole, Dorset
Phloretin	Sigma Chemical Company Ltd, Poole, Dorset
Siemens pump	Siemens, Erlangen, West Germany
Silicone oil 200/50 cs	Dow Corning Corporation, Midland, Michigan U.S.A.
Sutures, Vicryl and catgut	Ethicon Ltd, Edinburgh
Trypan blue	Sigma Chemical Company Ltd, Poole, Dorset
Tubes, polypropylene and polystyrene	Sarstedt Ltd, Leicester

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